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AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

IN SESSION IN

Baltimore, at the Johns Hopkins Medical School,

December 28 to 31, 1908, inclusive.

EDITED BY THE SECRETARY.

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

Johns Hopkins Medical School, Baltimore, December 28–31, 1908.

First meeting.

Monday afternoon, December 28.
Presiding officer: the President, John J. Abel.

ENTEROKINASE IN INFANCY.

By A. E. AUSTIN.

(*From the Medical Chemistry Laboratory of Tufts College and the Chemical Laboratory of the Medical Department of the University of Texas.*)

The object of this research was to learn whether enterokinase was found in infancy and, if so, at what age it appeared; furthermore, what effect, if any the time of the autopsy after death, the age of the infant and the disease from which it died had upon the amount found. It was necessary to rely upon extracts from the pancreas and duodenum obtained from the autopsy room, a method which had been found by Cohnheim and others to be satisfactory. As a means of determining the amount of digestion, the method of Loehlein with the casein solution of Weber was employed. Three series of experiments were carried out with cooked pancreas extract, cooked intestinal extract, and cooked pancreatic and intestinal extracts combined as controls, to each one of which an equal amount of the stock casein so ution was always added as a still further control. The results were as follows: (1) Extracts of the pancreas of infants, from 24 days of age onward, possess an active proteolytic ferment, appearing in the extract, at least partially, activated. (2) The extracts of the intestines of 9 of the 18 infants examined gave evidence of an enterokinase whose amount did not depend for variation on the

age, the period of autopsy after death (up to 24 hours), nor the disease from which the child died, among which were pneumonia, rachitis, empyema, heart disease and one case of enteritis. (3) No evidence of an antikinase was discovered.

THE INFLUENCE OF COLD STORAGE UPON FLESH.

By A. D. EMMETT and H. S. GRINDLEY.

(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois.)

The object of this study was to ascertain, by means of the chemical methods in use in this laboratory, something as to the nature of the changes which take place in flesh during cold storage, (*a*) in refrigerated beef, and (*b*) in frozen fowl. The results of the experiments show, in the case of uncooked beef, when allowances were made for the differences in fat content, that, during the 22 day period of cold storage, the meats lost almost no water, but during the 43 day interval they lost an average of 1.26 per cent. Further, by calculating the percentage constituents of the various cuts to the same water content as that of the corresponding fresh sample, the data show that for the first three weeks there was an increase in the soluble inorganic phosphorus; a decrease in the non-protein nitrogen, the nitrogenous extractives and the total soluble nitrogen; no change in the percentage of total protein, and, during the following three weeks, no increase, to any extent, in soluble inorganic phosphorus in the meats; but, on the other hand, the nitrogenous and non-nitrogenous extractives and also the total soluble and non-protein nitrogen made marked gains. The percentage of soluble non-coagulable protein remained practically the same as that in the fresh samples.

In the experiment upon boiling and roasting, it was found that the cold storage meats lost less on cooking; and that the resulting cooked meats were juicier; that they contained more soluble dry substance and more nitrogenous and non-nitrogenous extractives; that they were richer in soluble inorganic phosphorus; that the per cent of the total nitrogen was about the same as in the sample of the fresh cooked meat while that of the total soluble nitrogen was higher in the cold storage samples; that the percent-

age of the total protein was slightly lower, being on an average for all the tests, 26.45 per cent for the stored meats and 26.96 per cent for the fresh samples, and that the ratios of the non-protein nitrogen and the protein nitrogen of the meats were lower for the cold storage flesh, thus showing the influence of the increased percentage of nitrogenous extractives.

PHENOMENA OF ABSORPTION BY STRETCHED MUSCLE.

By WALTER E. GARREY.

(From the Laboratory of Physiology and Pharmacology of Cooper Medical College, San Francisco.)

A detailed study of the increase in weight of muscles which have been stretched and then immersed in physiological salt solution, shows that it is a function of the amount of stretching force, and of the time during which the weight has been suspended from the muscle. With heavy weights the effect is marked; for example: A muscle stretched for 4 hours by a 500-gram weight increases in weight 22 per cent when subsequently placed in a solution of sodium chlorid from which the control muscle increases only 3 per cent in the same time (20 hours). The results with small weights (50 grams or less) are variable and apt to be misleading unless the muscles are stretched for a considerable time. This is due to the fact that with small weights the effects are slight and that normal control muscles vary considerably. Distinct effects can, however, be obtained if the smaller weights act for a longer time.

The absorption curves show a sharp rise immediately after the immersion of the weighted muscle in physiological salt solution, but after the first hour or so, the rate of absorption suddenly changes and the curve assumes an entirely different form. Data at hand indicate that the rapid initial effects are due to osmotic differences, while the slower changes are due to another factor, probably chemical, and the result of formation of lactic acid (Gotschlich). Alterations in the weight of stretched muscles immersed in hypotonic and hypertonic solutions also indicate that more than one factor underlies the absorption phenomena.

The changes induced in a given time interval during the early periods of stretching are quantitatively less than those taking place later, that is, the changes are accelerative and, I would suggest, that they are autocatalytic. Autocatalysis explains beautifully certain phenomena of excitability and rhythmicity resulting from stretching.

THE DETERMINATION OF TOTAL SULPHUR IN URINE.

By F. W. GILL and H. S. GRINDLEY.

(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois.)

During the routine work connected with the determination of the total sulphur in a large number of urines by the Folin sodium-peroxid method, it was noticed that, more or less frequently, the resultant fusion, upon acidification with hydrochloric acid, evolved hydrogen sulphide gas. The Folin sodium-peroxid method, as applied to urines, does not, like the Osborne sodium-peroxid method as used for the determination of sulphur in air-dried foods and other dry substances, specifically include addition of sodium-peroxid to the hot fusion to insure complete oxidation. The nitric acid-potassium nitrate method of A. Konshegg was used to check the sodium-peroxid methods. This method gave upon an average 10.3 per cent more sulphur in human urines than did the Folin method and it gave 6.25 per cent more sulphur than did the original Osborne method as applied to urines. The evaporation and heating of urines with sodium-peroxid in a side-necked distilling flask, in such a manner as to collect all the volatile sulphur products, proved that volatile sulphur derivatives were evolved in connection with these operations. The research proved conclusively that there is a loss of sulphur attending the determination of this element in urines where the sodium peroxid methods are used.

THE DETERMINATION OF PHOSPHORUS IN FOODS, FECES AND URINE.

By F. W. GILL, J. B. PETERSON and H. S. GRINDLEY.

(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois).

The methods for the determination of total, inorganic and organic phosphorus in flesh were studied in detail. A thorough comparison of the (*a*) magnesium nitrate, (*b*) nitric acid (*c*) aqua-regia, (*d*) Kjeldahl, (*e*) hydrochloric acid and potassium chlorate, (*f*) nitric and hydrochloric acids and (*g*) direct ashing methods, for the oxidation of flesh preparatory to the precipitation of the total phosphorus by ammonium molybdate solution, demonstrated the fact that methods (*a*), (*b*), (*d*), (*f*), and (*g*) gave practically identica results in the hands of careful and thorough analysts. This study indicated clearly that a solution of barium nitrate in the presence of ammonium hydroxid or a solution of barium hydroxid alone, precipitates from an aqueous extract of flesh after the coagulable proteins have been removed, or from a solution of potassium di-hydrogen phosphate practically the same amount of inorganic phosphorus as does the Hart-Andrews method. The accuracy and economy of the nitric acid or the nitric-hydrochloric acid digestion methods for the determination of phosphorus in flesh led to the testing of these methods for the digestion of the urines previous to the precipitation of the total phosphorus with the ammonium molybdate. Check determinations of this nitric-hydrochloric acid method by several other methods of digestion proved that the oxidation of the organic matter was comp ete enough to convert all the phosphorus into a form precipitated by the ammonium molybdate solution. A comparison of the uranium acetate volumetric method for the determination of phosphorus in urines with the nitric-hydrochloric acid method demonstrated the fact that the former method invariably gave lower results than did the latter method.

The chemical constitution and the physiological effects of acids.

By Jacques Loeb.[1]

[1] See Loeb: *Biochemische Zeitschrift*, 1909, xv, p. 254.

Second Meeting.

Tuesday morning, December 29. Joint session with the American Physiological Society.

Presiding officers: The President of the American Physiological Society, William H. Howell, and the President of the American Society of Biological Chemists, John J. Abel.

THE GLOBULINS OF THE EGG-YOLK OF SELACHIANS.

By C. L. ALSBERG.

(From the Laboratory of the U. S. Bureau of Fisheries at Wood's Hole, Mass.)

The ovarian eggs of the skate, *Raja lævis*, Mitchell, contain a characteristic vitellin. In the eggs of the spiny dog-fish, *Squalus Acanthias*, Linn., previously studied, none could be found. As the skate is oviparous, and the dog-fish viviparous, this fact may have a biological significance.

THE PROTECTION TO ACETONITRILE POISONING BY THYROID FEEDING.

By S. P. BEEBE.

(From the Laboratory of Experimental Pathology, Cornell University Medical School, New York City.)

A series of experiments was made on mice to test the protective power of thyroid proteins against acetonitrile.

A standard Kahlbaum preparation of acetonitrile was used. The mice were fed on a uniform diet of cracker dust cakes for several weeks before the experiments were begun and various preparations of protein were employed.

We were unable to secure the uniform and invariable protection which Hunt has reported in an earlier paper. Our results show 60 per cent protection and about 40 per cent of either failure to protect or actual susceptibility.

FURTHER EXPERIMENTS ON TETANY PARATHYREOPRIVUS.

By S. P. BEEBE.

(From the Laboratory of Experimental Pathology, Cornell University Medical School, New York City.)

Further experiments in the production of tetany have given additional confirmation to the belief that the removal of the parathyroid is particularly responsible for the symptoms of tetany. These symptoms may be relieved by the hypodermic administration of parathyroid nucleoprotein, subject to the following conditions:

(1) Boiling the nucleoprotein solution or heating it to 80° for one-half hour completely destroys the activity of the nucleoprotein.

(2) The nucleoprotein is most active when freshly prepared and rapidly deteriorates when kept in solution or in suspension at refrigerator temperature. Freezing also destroys its activity although not so rapidly as room temperature.

(3) Tryptic digestion or the action of pepsin and hydrochloric acid for 48 hours injured, but did not completely destroy, the activity of the nucleoprotein.

(4) The nucleoprotein will relieve tetany if given by mouth, but is much more quickly and certainly effective when given subcutaneously or intraperitoneally.

(5) The nucleoprotein of the parathyroid when freshly prepared is equal to the whole gland in relieving the symptoms of acute tetany in dogs.

(6) The globulin is of no value in relieving tetany.

The relief of the symptoms by the intravenous injection of calcium salts may not be taken as proof that the symptoms are due primarily to a deficiency of calcium in the blood and tissues. Symptoms may be relieved also by injections of strontium salts. The symptoms caused by the intravenous administration of ammonia or xanthin are quite similar to those of tetany and may be relieved by an injection of calcium or strontium salts.

The total evidence leads to the conclusion that the symptoms are due to some poison which is produced as a consequence of a disturbed metabolism incident to the removal of the parathyroid.

ETABOLISM IN MAN WITH GREATLY DIMINISHED LUNG AREA.

By THORNE M. CARPENTER and FRANCIS G. BENEDICT.

(From the Chemical Laboratory of Wesleyan University.)

An experiment on a man whose left lung was completely obliterated but who was otherwise normal was made with a respiration calorimeter at Wesleyan University, Middletown, Conn. The experiment lasted six hours and during this period the carbonic acid elimination and oxygen consumption, water vaporization and heat production were determined. The pulse varied from 58 to 70 and the temperature was 36°.67. The naked weight was 47.3 kilos and the height, 1.69 meters. The total metabolism is low, but, when calculated on the basis of per kilo of body weight and compared with the results obtained from similar experiments on other subjects somewhat approximating the weight and state of nutrition of this subject, the results show that the metabolism was substantially at the normal level for thin persons of small body weight.

ON THE INTERNAL SECRETIONS OF THE THYROID.

By A. J. CARLSON and A. WOELFEL.

(From the Hull Physiological Laboratory of the University of Chicago.)

It is generally held that the internal secretion of the thyroid reaches the blood by way of the neck lymph. This view rests on the assumption that the colloid material within the tubules constitutes or contains the internal secretion of the gland. The only basis for the view that the internal secretions pass into the lymph appear to be histological—colloid being sometimes found in the tissue or lymph spaces.

(1) The main lymphatics for the thyroid leave the upper pole of the glands and join the main neck lymphatic trunk. In normal thyroids the lymphatics are relatively small, smaller than from the salivary glands of corresponding size. The flow of lymph from the normal gland is very slight, probably not exceeding 2 to 5 cc. in 24 hours.

(2) All forms of glandular hyperplasia (goitre) in the dog are accompanied by an increase in the size of the gland lymphatics similar to that of the gland blood vessels. There is a corresponding increase in the quantity of lymph flowing from the gland. This condition obtains even after the gland has become hematomatous. In goitre of the size of the kidney, the lymphatics are larger and the lymph flow is much greater than in the kidney. In large goitres the lymph production is probably from 50 to 150 cc. in 24 hours. By massage of the gland more lymph can be collected from a large goitre in two hours than is yielded by a normal gland in 5 to 7 days.

(3) Tests for thyroid secretions in the lymph. *A. Chemical.* All of our tests for iodine in the goitre lymph (quantities of 20 to 100 cc.) have been negative. *B. Physiological.* (a) Intravenous injection of goitre lymph in a normal dog produces a rise of temperature (2° to 3° F.), irregularity of the heart beat, and usually tremor of the skeletal muscles. These phenomena disappear within 10 to 20 hours. (b) Intravenous injection of goitre lymph in dogs under general anæsthesia causes a gradual depression of the blood pressure accompanied by a rapid, feeble and irregular heart beat. This is due partly to action on the vagi centers in the medulla. These phenomena suggest hyperthyroidism. (c) Hunt's acetonitrile method. The experiments have not yet yielded definite results. (d) The elimination of all the thyroid lymph in the fox and noting symptoms of thyroidectomy. Results so far are negative.

(4) While the colloid is a product of the secretory acini, it has probably no relation to the internal secretions of the gland that have physiological importance.

ON THE ORIGIN OF TAUROCHOLIC ACID.

By R. B. GIBSON.

(*From the Laboratory of Physiological Chemistry, Department of Physiology, University of Missouri.*)

The direct chemical relationship of cystine and taurine has been established by Friedman. Evidence of the relation of the taurine of the taurocholic acid in the bile to the cystine of protein is given by Bergmann, who fed cystein and cholic acid to a dog with a

biliary fistula and found the output of alcohol-soluble sulphur in the bile distinctly increased. Bergmann's observations were subsequently corroborated and extended by Wohlgemuth for rabbits. The well known experiments of Baumann and his pupils that cystein is conjugated with brom- or chlorbenzol and eliminated as mercapturic acid, afford a means of further demonstrating the origin of taurine in taurocholic acid. If brombenzol be administered, the resulting artificial cystinuria should deflect the cystine ordinarily oxidized to cysteinic acid and conjugated with taurocholic acid. The output and content of the bile in alcohol-soluble sulphur should then be considerably diminished as the result of the administration of brombenzol. Some preliminary observations were made, therefore, on the influence of brombenzol on the content of cat bile in alcohol-soluble sulphur. The cats received, as a rule, three injections subcutaneously of 0.6 to 1.0 cc. of brombenzol in 2.5 cc. of olive oil at 8 to 10 hour intervals; the animals were killed 5 to 8 hours after the last injection, and the alcohol-soluble sulphur determined as barium sulphate after fusion with sodium hydroxide and potassium nitrate with the usual precautions. The content of bile in taurocholic acid was found to be diminished over half as the result of the administration of the brombenzol. Thus per gram of bile, the alcohol-soluble sulphur as barium sulphate for normal cats was 0.0528, 0.0432, 0.0367, 0.0622, 0 0599, 0.0648, 0.0580, 0.0605, averaging 0.0548; for the brombenzol cats the figures are 0.0309, 0.0195, 0.0387, 0.0144, averaging 0.0259. While observations on a dog with a biliary fistula have not as yet been made, the results already presented indicate the direct origin of taurine from the cystine of protein and fit in with the experimental evidence given by Bergmann and by Wohlgemuth.

EXPERIMENTAL GLYCOSURIA.

By J. J. R. MACLEOD.

(*From the Laboratory of Physiology, Western Reserve University, Cleveland, O.*)

A brief review was given of recent work by the author on the mechanism of asphyxial glycosuria.[1]

[1] A full account of this work appears in the current volume of the *American Journal of Physiology*, (xxiii, p. 278, 1909).

No hyperglycæmia was found to follow asphyxiation in anæsthetized dogs when the liver is removed from the circulation by anastomosing the vena porta to the vena cava and ligating the hepatic arteries. The liver is, therefore, the sources of the reducing substance in asphyxia.

When the hepatic nerves are cut and an animal then asphyxiated (or curare injected) hyperglycæmia results. The asphyxial blood, therefore, acts directly on the liver cells and not through the intermediation of the nervous system.

By comparing the rate of glycogenolysis in incubated specimens of minced liver and blood in the presence of different gases, it was found that want of oxygen does not accelerate the process whereas excess of carbon dioxide does. This points to the excess of carbon dioxide rather than the deficiency of oxygen as the exciting factor in asphyxial blood. Other acid substances produced in the blood during asphyxia almost certainly reinforce the carbon dioxide in its glycogenolytic effect.

HEAT COAGULATION IN SMOOTH MUSCLE; A COMPARISON OF THE EFFECTS OF HEAT ON SMOOTH AND STRIATED MUSCLE.

By EDWARD B. MEIGS.

(From the Laboratory of Physiology in the Harvard Medical School.)

The striated muscle of the frog, when heated to 50° C., shortens greatly; whereas the smooth muscle of the same animal when similarly heated lengthens almost as markedly.

There is every reason to believe that the chemical and physical phenomena, which take place in the two tissues at temperatures of 50° and lower, are similar. In both cases irritability is lost at about 40°; irretrievably lost, if the tissue be kept for five or ten minutes at 50°. In both cases a marked whitening and opacity occur at 50°, and the proteins in the extracts of the two tissues are precipitated at 50°, provided the reactions of the extracts are made neutral. In both cases very considerable amounts of lactic acid are formed at temperatures between 40° and 50°; much more, it is true, in the striated than in the smooth muscle.

Finally, no considerable change in the weight occurs in either

tissue, if it be kept for 30 minutes at a temperature of 50° in 0.7 per cent sodium chlorid solution.

The changes which occur in the two tissues at temperatures above 50° are of an entirely different character. The shortenings which occur at these temperatures are by no means characteristic for muscle. They occur more rapidly and to a greater extent in catgut, connective tissue, elastic tissue, nerve, etc. They are more marked in muscle in which the proteins have been coagulated by two or three days' treatment with 70 per cent alcohol than they are in fresh muscle. They are more or less reversible and are always accompanied by a loss of fluid content and, consequently, of weight

The shortening of striated and the lengthening of smooth muscle between the temperatures of 40° and 50° C. may be explained in the following manner: The heating causes the production of lactic acid in both kinds of muscle. The presence of the acid causes the swelling of the sarcostyles in the one case, and of the cells in the other, at the expense of the interstitial fluids. The histological structure of the sarcostyle of striated muscle, on the one hand, and of the cell of the smooth muscle on the other, is of such a nature that swelling causes the former element to shorten and the latter to lengthen.

VEGETABLE AGGLUTININS.

By LAFAYETTE B. MENDEL.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*)

In continuing the study of hæmagglutinins earlier reported in the case of ricin by Osborne, Mendel and Harris,[1] it has been ascertained that agglutinins are widely distributed in various species of leguminous seeds.[2] In some cases, at least, they are non-toxic, thus indicating that the agglutinins may exist independent of the toxins. Soluble protein preparations obtained from some of the species of *Phaseolus* and *Pisum* afford very satisfactory non-toxic solutions for the demonstration of the

[1] Osborne, Mendel and Harris: *American Journal of Physiology*, xiv, p. 259, 1905.

[2] Cf. also Landsteiner and Raubitscheck: *Centralblatt für Bakteriologie*, xlv, p. 664, 1907.

hæmagglutination phenomena. Bloods from different species appear to show different degrees of reactivity with these agglutinins, blood from the rabbit, for example, responding more readily as a rule than that from the pig.

THE EXCRETION OF MAGNESIUM AND CALCIUM.

By LAFAYETTE B. MENDEL and STANLEY R. BENEDICT.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

When magnesium salts are introduced parenterally into animals, the elimination of magnesium takes place to a great extent through the kidneys within a relatively short period (48 hours). Some of the magnesium injected may, in part, be retained in the organism for a period exceeding two weeks. It is not excreted in increased amounts with the feces. Purgation is not observed after parenteral introduction of magnesium sulphate; if anything, the reverse effect may be noted. The increased urinary output of magnesium observed immediately after injection of its salts was regularly accompanied by an increase in the amount of calcium eliminated through the kidneys. There may be a decrease in the calcium excreted by the bowel. The elimination of magnesium through the kidneys after introduction of its salts appears to be more extensive in the rabbit than in the dog. The output of nitrogen and of chlorine was not appreciably affected by injections of magnesium salts.

When calcium salts are introduced into the circulation, calcium is abundantly excreted in the urine, particularly in the rabbit. There is, simultaneously, an increased output of magnesium by the kidneys.

Numerous other details will be published in a more elaborate report of the experiments.

PROTEIN METABOLISM IN DEVELOPMENT.

By J. R. MURLIN.

(From the Physiological Laboratory of the University and Bellevue Hospital Medical College, New York City.)

Experiments on pregnant dogs, lasting throughout the entire gestation period, were reported. In one of these, a nitrogen bal-

ance was kept in weekly periods. It was found, in agreement with the work of Hagemann, Ver Eckeand Jägerroos, that a minus nitrogen balance existed throughout the first four weeks and a progressively increasing plus balance throughout the last five weeks. Nitrogen and sulphur partitions were made on the urine on two days of each week in one case, and on one day of each week in the other. In a third case the dog was kept on a creatin-free diet during the first and last weeks of gestation and the first week post partum. The creatinin output was constant, but creatin appeared in the urine two days before parturition and reached a maximum on the fifth day after parturition. This favors the view that the involution process actually begins before the parturition and probably marks its maximum intensity (for the dog) on the fifth day thereafter.

The theoretical significance of the minus nitrogen balance in the early part of the gestation was discussed.

TOTAL (OR ENERGY) METABOLISM IN DEVELOPMENT.

By J. R. MURLIN.

(From the Physiological Laboratory of the University and Bellevue Hospital Medical College, New York City.)

In two of the pregnancy experiments (on the same dog) reported under the previous title, the dog was kept in the respiration apparatus on the third day previous to parturition and (together with the puppies) on the first day after parturition. From the first pregnancy one puppy was born; from the second, five. The dog on each of these days, and on one day three weeks after the first parturition, was kept at the same temperature and had eaten the same diet.

The total nitrogen and total carbon excreted, as well as the total energy metabolism calculated from these, are given in the table on next page.

The metabolism due to the pregnant condition is found by subtracting the total metabolism on the day of sexual rest from that on the third day before parturition in each case. Thus: $551.3 - 505.3 = 46$ cal. and $764.9 - 505.3 = 259.6$ cal. The extra

metabolism due to the pregnant condition proves therefore to be almost exactly proportional to the weight of the puppies at birth, thus: 46: 280:: 259.6: 1560 (nearly), or 164.3 cal. per kilogram with 1 puppy *in utero* and 160 cal. per kg. with five puppies *in utero*.

The differences obtained by subtracting the total resting metabolism of the mother dog from the total metabolism of the mother and puppies on the day after parturition are not quite proportional to the weights, probably for the reason that five puppy dogs helped to keep each other warm, thereby somewhat reducing the metabolism.

	DAY.	TOTAL N.	TOTAL C.	TOTAL CAL.		
	1908	*gms.*	*gms.*			
Third day before	VI/23	8.608	59.415	551.3	2	First pregnancy; one puppy born; weight 280 gms.
First day after	VI/27	8.455	65.855	640.6		
	VII/15	5.276	51.657	505.3		Sexual rest after lactation.
Third day before	XII/11	6.831	74.670	764.9	4	Second pregnancy; five puppies born; weight 1560 gms.
First day after	XII/15	8.389	100.620	1058.8		

Respiration experiments were performed on the individual puppy dogs of the second litter immediately after birth and before they had nursed. The respiratory quotient was found to be almost exactly 1, indicating the combustion of carbohydrate (glycogen).

On the pharmacological action of some phthaleins and their derivatives, with especial reference to their behavior as purgatives. By JOHN J. ABEL and L. G. ROWNTREE.

The secretion of hydrochloric acid in the stomach. By A. B. MACALLUM (with MISS M. P. FITZGERALD).

Third Meeting.

Wednesday morning, December 30. Joint session with the Biological Section of the American Chemical Society.

Presiding officer: The President of the American Society of Biological Chemists, John J. Abel.

THE RELATION OF MAGNESIUM AND PHOSPHORUS TO GROWTH IN THE FUNGI.

By HOWARD S. REED.

(From the Laboratory of Plant Pathology and Bacteriology, Virginia Agricultural Experiment Station, Blacksburg, Va.)

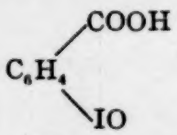
Magnesium and phosphorus, which are essential for the growth of fungi, have especial relation to the process of spore formation in *Aspergillus niger*. The ratio of these elements present in a good synthetic nutrient solution is conducive to good development of both mycelium and spores. Certain relations between the amounts of magnesium and phosphorus present bring about striking effects upon growth, as, for example, when the amount of phosphorus is reduced to certain fractional amounts, spore formation may be almost entirely inhibited without materially affecting the growth of the mycelium. These effects are believed to throw some light upon the relation of magnesium and phosphorus to the formation of oils and fats in the plant cell.

THE OXIDATION OF CARBON MONOXID.

By J. H. KASTLE.

(From the Division of Chemistry, Hygienic Laboratory, U. S. Public Health and Marine-Hospital Service, Washington, D. C.)

Attention was called to the importance of devising a method of oxidizing the carbon monoxid in combination with hemoglobin, with the view of restoring persons poisoned by this gas; and to certain reactions whereby we might hope to accomplish this oxidation. In this connection it occurred to the writer that possibly iodoso benzoic acid[1] might be employed to accom-



plish this oxidation. Some evidence has been obtained that this compound can oxidize carbon monoxid under certain conditions.

[1] See Meyer and Wachter: *Berichte der deutschen chem. Gesell.*, xxv, pp. 2632–2635, 1892.

Whether it can accomplish this oxidation in the animal body remains to be demonstrated. The few observations which have thus far been made on this subject are only preliminary to further studies which it is proposed to undertake.

THE FACTORS WHICH INFLUENCE THE DETERMINATION OF CREATININ.

By F. C. COOK.

(*From the Laboratory of Animal Physiology, Bureau of Chemistry, U. S. Department of Agriculture, Washington, D. C.*)

Several factors which influence the colorimetric method for determining creatinin as devised by Folin were studied. When more than 10 cc. of the alkali were used, lower results were obtained due to the effect of the alkali in dissolving the creatinin picrate compound. Thirty cubic centimeters of picric acid gave but slightly higher results than 15 cc.

The following factors tend to lower the results for creatinin: the dilution of the solution, the period of standing, the presence of coagulable protein such as egg-albumin, and, finally, the presence of proteoses and peptones.

Four methods, viz: the autoclave method of Benedict and Myers, the method of Grindley and Woods, the method of heating for four hours in a boiling water bath, and the method formerly employed at the chemical laboratory of Armour and Co. were compared. The most satisfactory results were obtained by using the autoclave method.

ENZYMES OF SOME LOWER FUNGI.

By ARTHUR W. DOX.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*)

The enzymes were prepared by growing pure cultures of the mold on a protein-free medium and dehydrating the mycelium by Albert and Buchner's method for "Acetondauerhefe." The Penicillium of Camembert cheese was used principally. It was found to contain a protease which digests casein, gelatin and Witte-peptone, but which is without action on fibrin, elastin, ovalbumin, vitel-

lin, edestin and excelsin. Its greatest activity is at the neutral point of methyl orange. It resembles Cohnheim's erepsin and the so-called glutinase. A hippuric acid splitting enzyme was also found which was quite active. Two green molds, *Penicillium chrysogenum* Thom and *Penicillium Roqueforti* Thom, both of which answer to Link's description of *Penicillium glaucum*, yielded in the one case 83 per cent hydrolysis and in the other case no hydrolysis at all. Attention is therefore called to the necessity of using definitely identified organisms. The numerous carbohydrate-splitting enzymes found in fungi by previous investigators were due, in part, at least, to a breaking down of carbohydrate contained in the fungus itself.

THE INDIRECT COLORIMETRIC ESTIMATION OF P_2O_5 WITH URANIUM ACETATE AND POTASSIUM FERROCYANIDE.

By R. B. GIBSON and CLARENCE ESTES.

(From the Laboratory of Physiological Chemistry, Department of Physiology, University of Missouri.)

The addition of an excess of uranium acetate solution and the determination of the uncombined uranium colorimetrically after decanting or filtering from the precipitated uranium acetate have given fairly satisfactory results, especially when only small amounts of phosphate are present. Ten cubic centimeters of the uranium phosphate solution ordinarily employed for the volumetric estimation are diluted up to 1000 cc. Nitric acid digestions or $NaOH + KNO_3$ fusions are neutralized, acidified with acetic acid and the volume made up to 100 cc. About 5 cc. of an accessory solution (containing only a fifth the usual proportion of sodium acetate) are added to each of duplicate 10 or 20 cc. portions of the phosphate solution. The diluted uranium solution in 15, 25 or 50 cc. amounts, according to the probab e content of phosphate, is added, and the volume is made preferably to twice that of the uranium acetate emp oyed. After standing until precipitation has occurred, usually several hours, 20 cc. aliquots of the filtered or decanted solution are pipetted off, and 0.5 to 1.0 cc. of a 10 per cent potassium ferrocyanide solution added according to the amount of uranium acetate present. The

red-brown color is completely developed in 10 to 15 minutes. The solution should be diluted, after adding the ferrocyanide, up to 100 cc. A blank digestion or fusion should be employed in making the contrast, since nitrates somewhat increase the intensity of the color obtained. The P_2O_5 content of the aliquot of the fusion taken is determined by multiplying 0.00005 g. by the difference between the number of cubic centimeters of uranium acetate solution added and of x in the colorimetric proportion, Unknown (fixed): Known:: Uranium acetate solution added in cc.: x cc. Results obtained, when known amounts of P_2O_5 (2.5 mgm.) have been added to fusion mixtures, have indicated an accuracy by the above procedure to tenths of a milligram. Determinations on stock solutions (0.2 to 5.0 mgm. in the aliquots precipitated) alone have been accurate to 0.0001 mgm. The addition of more sodium acetate than is above indicated will inhibit the appearance of the ferrocyanide reaction. Ammonium sulphate has little or no influence on the color developed in the presence of nitrates. As a rule there is no interference with the determinations because of precipitation of the uranium ferrocyanide compound. Color readings should be made within a few hours after the addition of the ferrocyanide.

AN APPARATUS FOR THE FILTRATION OF LARGE AMOUNTS OF SERUM, TOXINS, PROTEIN SOLUTIONS, ETC., THROUGH A BERKEFELD BOUGIE.

By R. B. GIBSON.

(From the Research Laboratory of the Department of Health of the City of New York).

The china-lined iron cylindrical chamber of the Berkefeld filter battery of three large bougies is obtained without the latter. The small nickeled metal chamber for the single large bougie commonly used for household purposes is also purchased. A stopcock is placed between the large and small metal chambers so that the two may be screwed together. When the large cylinder is bolted into place on the wall, it serves as a reservoir or container for the solution to be filtered; the smaller chamber, which can be shut off from the larger by the stopcock above mentioned, contains the large laboratory bougie, The bougie may be dry

sterilized apart from the apparatus and replaced at any time during the filtration if the connection between the reservoir and the filter chamber is cut off by turning the cock. Air from a convenient source of pressure, as a tank or bicycle pump, etc., is supplied into the larger chamber through a cock inserted into the opening in the removable cover through which the battery of filters were arranged to discharge. The filter candles when cleaned may be plugged with cotton and dry sterilized. The filtered solution is conducted with sterile rubber tube connections into sterilized bottles; when filled, these are replaced with the customary precautions. With the above described apparatus, there is but little of the material left unfiltered, loss is therefore minimal, the filtration is rapid and, when once started, requires but little attention. Sterilization only involves the handling of the filter candle. The cost is relatively slight, the essential parts being already on the market as popular household and industrial appliances. Filters, as above described have been used for the past two years at the Research Laboratories of the Department of Health of the City of New York, and in a proprietary serum laboratory in the same city. Another is in use in the Pathological Laboratory at the University of Chicago.

THE DESTRUCTION OF BODY-PROTEIN IN FEVER.

By PHILIP A. SHAFFER.

(From the Department of Experimental Pathology, Cornell Medical School, New York City.)

A brief report of metabolism experiments upon human subjects of typhoid fever. The results show that the loss of body-protein in fever, as indicated by the nitrogen balance, may be greatly retarded, and perhaps wholly prevented, by the intake of diets of high caloric value, and containing a large amount of carbohydrate. Carbohydrates exert a strong protein-sparing effect upon the metabolism in fever.

THE ANTAGONISTIC ACTION OF AMMONIUM AND CALCIUM SALTS. A CONTRIBUTION TO THE SUBJECT OF ACIDOSIS.

By C. VOEGTLIN and I. KING.

(From the Laboratory of Pharmacology and the Medical Clinic, Johns Hopkins University.)

The large output of ammonium salts in the urine, as well as the high ammonia content of the blood, in clinical conditions involving acidosis, suggested that these salts might play an important rôle in producing the symptoms of these diseases. The intravenous injection of ammonium salts of lactic, hydrochloric, and β-oxybutyric acids produced symptoms of acid intoxication. Injection (intravenous) of calcium salts completely antagonized the toxic action of the ammonium salts.

In order to determine if the ratio of ammonia to calcium was changed in acid intoxication, several organic and inorganic acids were fed to dogs, and the ammonia and calcium determined in the tissues. The ratio was found to be changed decidedly from the normal and always in favor of the ammonia constituent.

Investigation of the other alkalies (sodium, potassium, magnesium) of the tissues and blood is in progress.

While we do not consider that the changes in the alkalies are the only causative factors in acid intoxication, still we think that they have an important bearing on the subject.

CONCERNING THE SUPPOSED HYDROLYTIC ACTION OF PLATINUM BLACK.

By W. E. GROVE and A. S. LOEVENHART.

(From the Pharmacological Laboratory of the University of Wisconsin.)

In 1896 Rayman and Sulk studied the hydrolysis of cane sugar by finely divided platinum and claimed that some hydrolysis occurred. In 1900 Sulk found that finely divided metals inhibit the hydrolysis of amyl acetate by water. Since the appearance of the work of Bredig and von Bernack in 1899, in which the authors drew a close analogy between the colloidal metals and the enzymes, attempts have been made, especially by Neilson, to show that platinum black can effect the hydrolysis of those

substances ordinarily hydrolyzable by enzymes, viz: ethyl butyrate[1] and starch.[2] In his experiments Neilson used such large amounts of platinum, and the hydrolysis recorded by him was so slight, that there was apparently no justification for his conclusions that the platinum acted catalytically. Thus in most of his experiments he used more platinum than ethyl butyrate and in one of his experiments with starch *fourteen times as much platinum as starch was employed.* Such experiments obviously can throw no light on the *catalytic* action of platinum. In our work, the platinum black employed was extremely active in the catalysis of hydrogen peroxide and there is every reason to believe that it was in every way about as active a preparation as can be made. It was very finely divided and very black. One hundred cubic centimeters of a 1 per cent starch paste and 20 mgm. of platinum were placed in a flask and frequently agitated. After one month at 38° C. no hydrolysis could be determined. We conclude that platinum black cannot catalytically effect the hydrolysis of starch. Using about equal weights of platinum black and ethyl butyrate we have confirmed Neilson's observations that there is some acid production. When the quantity of the platinum is reduced to one-tenth or one-twentieth of the weight of ethyl butyrate employed, we have found no production of acid. We are inclined to believe that the acid production observed when larger amounts of platinum are used is due to oxidation by the oxides of platinum contained in platinum black—there is certainly no evidence that the platinum acts catalytically. More work must be done before we can draw the important conclusion that platinum black can induce hydrolysis.

THE DIASTATIC ENZYME OF RIPENING MEAT.

By A. W. PETERS and H. A. MATTILL.

(*From the Zoölogical Laboratory of the University of Illinois*).

Samples of lean meat, taken at the time of slaughtering, were cut into pieces of 20 grams weight, rinsed in saturated thymol water and then in distilled water, and finally preserved in steril-

[1] *Amer. Journ. of Physiol.*, x, p. 191, 1903.
[2] *Ibid.*, xv, p. 412, 1905.

ized test tubes. At intervals of a day or two, the ripening or autolyzing muscle was tested for its diastatic power by incubating it under toluol-thymol with starch solution and estimating the resulting sugar. All necessary blanks and controls were conducted parallel with the test experiments. The results from these samples and from others purchased in the market at random showed that so long as ripening muscle is in an edible condition the *sugar thus obtained* by a fermentation test will be considerable in amount and that during the later period of autolysis, when the meat has become inedible, the sugar recovered is comparatively small, finally diminishing to nothing. Probably the data do not represent the absolute diastatic power of autolyzing muscle but rather the excess of the diastatic over the negatively operating factors. This subject is under investigation by one of the authors (Peters).

THE ADSORPTION AND PARTIAL PURIFICATION OF CATALASE FROM LIVER.

By A. W. PETERS and H. W. STEWART.

(From the Zoölogical Laboratory of the University of Illinois.)

A catalase-containing aqueous extract of liver is treated with sodium phosphate and zinc sulphate. The precipitated zinc phosphate is found heavily charged with catalase which is removed with sodium phosphate and further purified by the same process of adsorption. With the observance of detail the final product represents a considerable degree of concentration and of purification. Complete adsorption of catalase by zinc phosphate from a not too concentrated solution is easily attained, thus showing the strong affinity between these two substances. The prepared catalase gives strong xanthoproteic, and practically negative Millon and biuret reactions. The most abundant precipitation reactions were obtained with phosphotungstic and with tannic acids. It is inactivated by boiling, by hydrocyanic acid, hydrogen sulphide and mercuric chloride, but a precipitate with mercuric chloride from the original aqueous extract long remains active. A given quantity of catalase-bearing zinc phosphate can be exhausted with hydrogen peroxide so that after washing,

fresh application of hydrogen peroxide produces no further evolution of oxygen. By the Kjeldahl process nitrogen is always obtained, the quantitative variation of which is still under study. Adsorption by other materials was found inferior to the zinc phosphate process.

ESTERIFICATION OF THE BILE ACIDS.

By ISAAC KING PHELPS.

(From the Laboratory of the Medical Department of George Washington University, Washington, D. C.)

A report of the extension of the earlier studies of esters to the isolation and identification of the bile acids.

ESTIMATION OF TOTAL SULPHUR.

By ISAAC KING PHELPS.

(From the Laboratory of the Medical Department of George Washington University, Washington, D. C.)

An attempt is in progress to separate sulphate ions completely as barium sulphate, which is then purified from contaminating substances by use of the Munroe crucible.

A STUDY OF NYLANDER'S REACTION.

By M. E. REHFUSS and P. B. HAWK.

(From the Laboratory of Physiological Chemistry of the Department of Medicine of the University of Pennsylvania.)

Bechold's claim that Nylander's test gives a negative reaction with urine containing sugar when mercuric chloride or chloroform is present was first investigated. We found no inhibitory action in the case of mercuric chloride and none in the case of chloroform provided the urine was heated to boiling for a period of five minutes previous to making the test. The findings in this connection were made previous to the report of Zeidlitz on the matter.

In addition to the above points the test was investigated from the following standpoints: (*a*) Most satisfactory method of performing the test; (*b*) Its delicacy; (*c*) The effect of tempera-

ture upon the reaction; (*d*) Interfering substances; (*e*) Its clinical value.

CONCLUSIONS.

I. The most satisfactory method of performing the test entails the heating of the combined urine and reagent for *five minutes in boiling water*. The practice of heating over a free flame is apt to lead to the drawing of erroneous conclusions.

II. The test is not available for the detection of less than 0.08 per cent of dextrose.

III. The length of time necessay to heat on a water-bath varies with the temperature. For example, a temperature of 57° C. must be maintained for 23 minutes to secure the same result that a temperature of 75° C. will yield in 5 minutes.

IV. Protein does not interfere to any extent with the reaction unless present to the extent of two per cent or over.

V. Such urinary constituents as urea, uric acid, creatinine, phosphates, and urates do not interfere with the test even when present in large excess. Tyrosine, leucine and cholesterol also give negative results. The urine from a case of cystinuria also failed to give a positive reaction.

VI. In the examination of over seven hundred urines there were only two instances in which Nylander's reaction was positive and Fehling's test negative. In Case I a very marked reduction occurred for no apparent reason and in Case II there was a very slight positive reaction.

VII. We consider that Nylander's reaction is a valuable confirmatory test and not useless as Pflüger claims it to be. We believe, as Kestermann has suggested, that any protein-free urine of acid reaction which gives a negative Nylander's reaction may safely be said to be *sugar-free* in a clinical sense.

THE ANTI-PUTRESCENT EFFECTS OF COPPER SALTS.

BY ALFRED SPRINGER, SR., AND ALFRED SPRINGER, JR.

(*From the Laboratory of Dr. Alfred Springer, Cincinnati, O.*)

The remarkable behavior of a Cincinnati certified milk, in not becoming putrescent, aroused the suspicion that it contained some antiseptic substance. After a long investigation, variable

minute quantities of copper were found in the bottles examined. In tracing the origin of the copper salts, it developed that the boiler compound (used on account of the hard water) primed or foamed over, thus contaminating the sterilizing cloths, pails and other utensils. Check experiments with and without the addition of copper salts were then made on a milk caught direct in broad mouthed bottles. It was shown that copper salts are selective in their actions, particularly retarding or inhibiting the putrescent bacteria, such as: The *Proteus vulgaris*, *Mirabilis*, *zenkeri* and *Clostridium fœtidum*. Unless considerable quantities of copper salts are used, the lactic bacteria are not greatly suppressed; consequently the milk will retain its sweet odor although the acidity contents may be high enough to curd it. Molds, such as *Penicillium glaucum*, *Aspergillus niger*, *Eurotium repens* and others are not inhibited by the copper salts and grow profusely on the certified milk. Experiments with copper salts in blood and egg albumin, meat, milk, and sewage solutions clearly demonstrated their anti-putrescent properties.

A DISTURBING FACTOR IN BARFOED'S TEST.

By WILLIAM H. WELKER.

(*From the Laboratory of Physiological Chemistry, Department of Medicine, University of Pennsylvania.*)

In applying Barfoed's test to the products of the hydrolysis of starch (obtained by boiling starch paste with hydrochloric acid), it was found that instead of obtaining the typical glucose reaction, a greenish white precipitate formed. Previous to the application of this test to the hydrolytic products of the starch, the solution was rendered alkaline with sodium hydroxid and then slightly acidified with acetic acid. A study of the cause of this interference, showed that sodium chlorid in water solution when boiled with Barfoed's reagent formed a similar precipitate. Sodium chlorid, added to a pure glucose solution, also gave with Barfoed's test a precipitate resembling the former ones.

A series of tests was made to determine the concentration of sodium chlorid which would interfere with the Barfoed test in glucose solutions of various strengths. The tests were carried out as follows:

5 cc. glucose solution of twice the concentration desired in the final mixture.
4 cc. NaCl solution of 2½ times the concentration desired in the final mixture.
1 cc. Barfoed's reagent.

This mixture was heated to boiling and boiled for 30 seconds.

The Barfoed reagent used was made up according to the directions of Barfoed[1] in his original paper, i.e., 5 cc. of 38 per cent acetic acid added to 200 cc. solution of copper acetate (1 part copper acetate to 15 parts water). The results of these tests showed that the presence of a very small percentage of sodium chlorid prevents the typical Barfoed reaction for glucose.

The nature and composition of the greenish white precipitate that forms in the presence of sodium chlorid has not yet been investigated.

Surface tension as a factor in the distribution of salts in animal and vegetable cells. By A. B. MACALLUM.

Fourth Meeting.

Wednesday afternoon, December 30. Joint session with Section K of the American Association for the Advancement of Science, and with the American Physiological Society and the Society of American Bacteriologists.

Presiding officer: The chairman of Section K of the American Association for the Advancement of Science, William H. Howell.

General papers, giving a résumé and the present status of the subjects treated, were presented, as follows:

Anaphylaxis. By M. J. ROSENAU. (Will be published in the *Archives of Internal Medicine*).

The physiological significance of creatin and creatinin. By LAFAYETTE B. MENDEL. (*Science*, xxix, p. 584, 1909).

The venous pulse. By ALBION W. HEWLETT. (*Science*, xxix, p. 515, 1909).

Fifth Meeting.

Thursday morning, December 31.

Presiding officer: The President, John J. Abel.

[1] Barfoed: *Zeitschr. f. analyt. Chem.*, xii, p. 27, 1873.

THE INFLUENCE OF THE ISOMERS OF SALICYLIC ACID ON METABOLISM.

By E. W. ROCKWOOD.

(From the Chemical Laboratory, University of Iowa.)

Variations in the urinary output were determined while the subjects were on a purin-free diet, particularly of the nitrogenous constituents. The ortho compound (salicylic acid) increases the uric acid while the total nitrogen, creatinin, phosphoric acid and ammonia are unaffected. The meta and para oxybenzoic acids do not have any action upon the amount of uric acid eliminated, nor the other compounds determined. The hypothesis is advanced that the uricolytic ferment is inhibited.

A STUDY OF THE METABOLISM OF A BREAST FED INFANT, WITH SPECIAL REFERENCE TO THE AMMONIA COEFFICIENT.

By S. AMBERG and W. P. MORRILL.

(From the Pharmacological and Obstetrical Departments of the Johns Hopkins University.)

The nitrogen metabolism and the partition of the nitrogen in the urine of a perfectly normal breast fed infant about four weeks of age and of about 4600 grams body weight was studied in two large periods of 6 days, each subdivided again in shorter periods of 48 hours. During the first period the infant received on an average 11.6 grams protein, 36.7 grams fat and 46.0 grams sugar *pro die;* during the second 3.6 grams protein, 19.7 grams fat and 35.1 grams sugar. The diet of the second period seems to have approached an "Erhaltungsdiat." Comparing the results obtained with those of other authors, it seems that the retention of nitrogen expressed in per cent of the nitrogen intake, as well as in per cent of the resorbed nitrogen, decreases as the infants advance in age from the first week to about the second or third month. With regard to the partition of the nitrogen in the urine it was noted that the creatinin nitrogen participated during the first period with an average of 2.3 per cent in the total nitrogen, during the second period with an average of 3.9 per cent. During the last 48 hours, where the amount of protein was the lowest,

this figure rose to 4.5 per cent. The average percentage of the uric acid nitrogen was 3.1 during the first, and 8.2 during the second period. The ammonia nitrogen rose from an average of 8.1 per cent to one of 18.3 per cent, in spite of the fact that, during the second period, the total amount of fat of the diet was lower than during the first periods. From the results obtained and from data recorded in the literature, the probability suggests itself that the ammonia coefficient—as far as it is influenced by the fat or protein content of the food—is not so much dependent on the absolute amounts of fat or protein as on the ratio between the fat and protein (F: P), at least within certain limits. Thus, to an average ratio F: P of 3.2:1 during the first period, corresponded an ammonia coefficient of 8.1 per cent, while with an average ratio F: P of 5.5:1 during the second period an ammonia coefficient of 18.3 per cent was noted.

ON NUCLEIC ACIDS.

By W. A. JACOBS and P. A. LEVENE.

(From the Rockefeller Institute for Medical Research.)

In a previous publication[1] we have shown that, on hydrolysis of inosinic acid with 5 per cent sulphuric acid as 50° C., a point is reached after 4 days at which the original lævo-rotatory power of the solution changes to a constant dextro rotation. From this solution we were able to isolate a crystalline barium salt of a pentose-phosphoric acid which possessed strong reducing properties. On alkaline hydrolysis, we showed that phosphoric acid was split off from the molecule without free hypoxanthin or free sugar appearing in solution. From this solution we obtained a silver compound of a complex consisting of a pentose and hypoxanthin. In our recent experiments we have succeeded in isolating the pentose-purin complex in pure crystalline condition. It showed no reducing power. From these facts we consider it conclusively proved that in the inosinic acid molecule, the phosphoric acid is bound on one of the hydroxyls of the pentose, and the hypoxanthin is linked to the aldehyde group of the sugar in a glucoside arrangement.

[1] *Berichte der deutsch. chem. Gesell.*, xli, p. 2703, 1908.

We believe it very likely that the nucleic acids are built up of groups, nucleotids, similar in composition to the inosinic acid, which are joined together as the phosphoric acid radicals in the polyphosphoric acids. From the yeast nucleic acids, we have isolated a body analyzing as a tetranucleotid in which the sugar is a pentose, and the bases are adenin, guanin, uracil and cytosin.

THE ABSORPTION OF PHENOL FROM THE ALIMENTARY CANAL.

By PAUL HANZLIK and TORALD SOLLMANN.

(From the Laboratory of Pharmacology, Medical Department of Western Reserve University, Cleveland.)

When concentrated phenol is introduced into the stomach or intestine of dogs or cats, in the dose of 1 gram per kilogram, the absorption starts with great rapidity. About 38 per cent, on the average, is absorbed within five minutes. When this point is reached the absorption is practically arrested, the remaining 60 per cent being unabsorbed even after five hours. The arrest of absorption was investigated along the following lines, with negative results: (1) It is not due to corrosion, for 5 per cent solutions, which should be less corrosive, are absorbed even more imperfectly, the absorption amounting on the average to 22 per cent. (2) It is not due to strictly local injury, for the introduction of phenol into a ligated loop of intestine lessens the absorption from a second fresh loop. (3) It is not due to low blood pressure, for animals whose initial blood pressure is low absorb as well as those whose initial blood pressure is high. Considerable absorption occurs even in the absence of circulation, i. e., in dead animals. (4) It is not due to reëxcretion of phenol into the intestine, for the amount so excreted is very small. (5) It is not due to a greater solubility in the gastric contents, for the excretion is not increased by distending the stomach with water.

The investigation is being continued in the hope of furnishing a positive explanation of the phenomenon.

THE LARGE WHITE OR SOAPY KIDNEY.

By OSKAR KLOTZ.

(From the Laboratory of the Royal Victoria Hospital, Montreal.)

The frequent observation has been made that the large white kidney, which appears fatty, really contains less fat than a normal organ. Others have shown that the large white kidney contains a large amount of myelins, and that these substances lead to a white appearance of the organ. In this analysis, it was found that the myelin bodies could be isolated in a pure state. The kidney substance was obtained immediately after death and was first extracted with ether. The residue, which contained the myelin forms, was then extracted with alcohol, into which the myelins passed in solution. These myelins were then precipitated with an excess of chloroform and then collected and analyzed. This material, which gave the reaction of fatty acid compounds, proved to be a compound of oleic acid with a very small quantity of another fatty acid, too small in quantity to allow determination. The bases present were potassium and sodium. The results were controlled in every stage of the work with the polarizing microscope which proved a very useful instrument in demonstrating the presence of the myelin bodies in each process of the examination. After the isolation of the oleic acid, it was found that the combination with the bases obtained gave myelin bodies identical with those in the original material. It is thus shown that the fatty substances present in the large white kidney are present in the nature of soaps and are not readily detected by ordinary staining methods.

THE MEASUREMENT OF THE ALKALI RETENTION OF THE KIDNEY.

By L. J. HENDERSON and H. M. ADLER.

(From the Laboratory of Biological Chemistry of the Harvard Medical School.)

Urine is treated with potassium oxalate, filtered, diluted and titrated with $\frac{N}{10}$ NaOH, using as indicator neutral red. The end point is found by matching the color with that given by a phosphate solution, Na_2HPO_4, 0.0085 molal, NaH_2PO_4, 0.0015 molal,

which possesses the same reaction as normal blood, $(\frac{+}{H}) = 0.4 \times 10^{7}$.

The result of this titration, plus the ammonia of the urine, measures the alkali retention.

ON IONIC EQUILIBRIA IN THE ANIMAL ORGANISM. I. THE IONIZATION CONSTANTS OF β-OXYBUTYRIC ACID AND ACETACETIC ACID.

By L. J. HENDERSON and K. SPIRO.

(From the Laboratory of Physiological Chemistry of the University of Strassburg.)

Measured with indicators, the ionization constants of the diabetic acids have been found to be approximately:

$CH_3—CHOH—CH_2—COOH$	2×10^{-5}
$CH_3—CO—CH_2—COOH$	1.5×10^{-4}

These results indicate that the amount of alkali removed from the body by β-oxybutyric acid may vary greatly with normal variations in the acidity of the urine.

ON THE COMPOSITION OF DILUTE RENAL SECRETIONS.

By A. B. MACALLUM and C. C. BENSON.

(From the Physiological Laboratory of the University of Toronto.)

The process of urine formation postulated in Ludwig's theory involves filtration from the blood plasma by the glomeruli and reabsorption of water and some other constituents of the filtrate by the convoluted tubules. The filtrate, therefore, before reabsorption takes place, should contain the salts of the plasma in the same relative proportions in which they obtain in the plasma. If now reabsorption could be prevented or greatly lessened, the resulting renal product should, in the relative proportions of sodium, potassium, calcium, magnesium, parallel the inorganic portion of the blood plasma. Such a diminution of reabsorption, if the latter occurs at all, must take place when great quantities of water are ingested, for the secretion formed by each kidney may be as much as 12 cc. per minute and the fluid secreted may

be so dilute as to give as high a Δ as $-0.08°$ C. An analysis, however, of these dilute secretions shows that they are not at all filtrations, that the secretions from period to period, each of 10 minutes, vary very widely in their inorganic composition from each other and from the blood plasma. It was further found that when the potassium excretion varied, the variations did not coincide with those ascertained to occur in the case of the chlorine in the periods. The results show that the elimination of the salts is a true secretory, not a filtration process and that the activity of the secretory structures is different for each inorganic constituent of the urine, that is, secretion of the salts is not a single act on the part of the epithelium concerned but a series of acts apparently independent of each other.

POST MORTEM GLYCOGENOLYSIS.

By J. J. R. MacLEOD.

(From the Laboratory of Physiology, Western Reserve University, Cleveland, O.)

Some preliminary experiments were reported dealing with the distribution of glycogen in the different lobes of the dog's liver. In two experiments it was found that the distribution is quite uneven.

The percentage of glycogen in a liver, removed immediately before death, is often slightly less than in that removed immediately after death, probably because, in the former case, there is more blood in the liver.

The rate of *post mortem* glycogenolysis was studied in two ways: A—By leaving the liver in the body and removing pieces for glycogen estimation at definite periods after death; B—By incubating mixtures of minced liver and blood in the incubator. By the first method, it was found that glycogenolysis is quite slow immediately after death but afterwards becomes very rapid. It therefore takes some time after death for the glycogenolytic process to become active. The average percentage glycogenolysis in all the observations (both A and B) in one hour was found to be 30.

In several experiments in which the great splanchnic nerve was stimulated, after depriving the liver of its portal blood supply

by anastomosing the portal vein into the vena cava, the average rate was found to be about 70. This result lends support to the view that there are glycogenolytic fibers in this nerve which act independent of the vasomotor fibers.

THE DETERMINATION OF IODIN IN PROTEIN COMBINATIONS.

By LOUIS W. RIGGS. (By invitation.)

(From the Laboratory of Experimental Pathology, Cornell University Medical College, New York City.)

The author suggested improvements of Baumann's process.

The oxidation of a part of the iodin to iodate during the fusion, and the necessity of a subsequent reduction was demonstrated. Without such reduction frequently 20 per cent or more of the iodin may be lost.

Carbon tetrachlorid is used to extract the iodin instead of chloroform or carbon disulfid.

Slim 10 cc. Nessler tubes are used for the color comparison. A difference of 0.01 mgm. is readily detected by this process.

THE ABSORPTION OF IODIN BY THE THYROID GLANDS IN DOGS.

ELEANOR VAN ALSTYNE and S. P. BEEBE.

(From the Laboratory of Experimental Pathology, Cornell University Medical College, New York City.)

A number of thyroid glands of dogs were analyzed in order to compare the absorptive capacity of thyroids in dogs of different ages.

The results show that in young animals, there is a maximum capacity of iodin absorption which diminishes with age and which in the old dogs may be *nil.*

THE IODIN CONTENT OF HUMAN THYROID GLANDS.

By LOUIS W. RIGGS and S. P. BEEBE.

(From the Laboratory of Experimental Pathology, Cornell University Medical College, New York City.)

The analyses of a number of normal human thyroid glands show but slight variation in the iodin content, the average quan-

tity being 0.33 mgm. per gram of fresh gland. Seven out of ten glands gave between 0.33 and 0.39 mgm. while the other three gave respectively 0.20, 0.26, and 0.43 mgm.

Several apparently normal glands gave a much higher iodin content, but as the clinical histories of these cases suggested the administration of iodin, the results were not included in the foregoing averages.

Seven glands, from cases of exophthalmic goitre in which the patients had *not* been given iodin, yielded an average of .08 mgm. of iodin per gram of fresh gland. Six of the seven gave a minimum of 0.06 and a maximum of 0.08, while the remaining one gave 0.16 mgm.

Other pathological thyroids gave large and exceedingly variable quantities of iodin.

Sixth Meeting.

Thursday afternoon, December 31.

Presiding officer: The President-elect, Otto Folin.

THE INFLUENCE OF CHLORAL HYDRATE ON SERUM ANAPHYLAXIS.

By EDWIN J. BANZHAF and L. W. FAMULENER.

(From the Research Laboratory of the Department of Health, New York City.)

In a preliminary communication, we reported that choral hydrate, when given in sufficient amounts intramuscularly, protected sensitized guinea pigs from the second injection of serum when given intraperitoneally. Further work has fully confirmed our earlier observations.

Choral hydrate, injected directly into the heart, protects sensitized guinea pigs from the second injection of serum into the heart and also protects sensitized guinea pigs from the second injection directly into the brain.

HUMAN PANCREATIC JUICE.

By HAROLD C. BRADLEY.

(From the Laboratory of Physiological Chemistry, Department of Physiology University of Wisconsin, Madison, Wis.)

Juice obtained after the removal of a pancreatic cyst was collected in twenty-four hour periods. It presented only a frac-

tion of the entire output of the pancreas since the patient was able to digest with perfect ease a regular ward diet of mixed food, gained rapidly in weight and strength, and showed no inability to handle considerable amounts of fat. For these reasons we assume that the portion of the secretion draining was normal juice. Ten cubic centimeters of it required about 8 to 10 cc. of $\frac{N}{10}$ acid to neutralize it. Of this only 0.4 cc. was as sodium carbonate; the bulk of the alkalinity was sodium bicarbonate. The specific gravity averaged 1010 at 20°. No rennin, invertase, or lactase could be demonstrated. Amylopsin was always present and rather uniform in amount. Trypsinogen was regularly demonstrable after the addition of dog's enterokinase solution. In nearly half of the samples trypsin itself was also present, and in the remainder, it regularly developed in two weeks, though the samples were saturated with toluol and in some cases chloroform also. Lipase was present in varying amounts. Its fluctuations bore no relation to diet, change in alkalinity, nor fluctuations of the tryptic content.

The lipase was made the subject of more detailed examination; the effects of bile salts, dilution, the addition of various salts to the digestions, shaking, and change of temperature were noted. Continuous shaking in a machine was found to inhibit the digestion markedly. A temperature of 20° to 30° was found to be optimum under the conditions of these experiments; but a temperature of 0° was much better than one of 40°. At 50° digestion was markedly inhibited while at 60° there was none. It is believed that the rapid slowing of the fat digestion above 30° is due to the increasing solubility of the products of digestion—especially in this case the oleic acid.

UROCANIC ACID IN A PANCREATIC DIGEST.

By ANDREW HUNTER. (By invitation.)

(From the Physiological Departments of Leeds University, England, and Cornell University, Ithaca.)

From a very long-continued (7 months) pancreatic digestion of casein a substance was isolated, which has not hitherto been observed under such conditions. By its elementary composition, crystalline form, melting point, and the characteristic appearance

of its nitrate, it was identified with the urocanic acid found by Jaffe and Siegfried, as a rare constituent of the urine of dogs. It was obtained in very small quantity (1.8 gram impure acid from about 400 grams of casein). A preliminary study of its reactions, and a consideration of the conditions under which I obtained it, leads me to believe that it is a derivative of histidin, and not, as Siegfried suggests, of the purin bodies. Probably it owed its origin in this instance not directly to tryptic action, but rather to secondary reactions taking place during the unusually prolonged digestion. This is rendered the more probable by the fact that arginin and histidin were entirely missing in the mixture of products.

THE ACTION OF SALTS USED AS FERTILIZER ON PLANT ENZYMES.

By M. X. SULLIVAN. (By invitation.)

(From the Bureau of Soils, U. S. Department of Agriculture, Washington, D. C.)

It has been found that potassium sulphate, even in dilute solution, has a retarding effect on the oxidizing action of wheat roots while sodium nitrate and mixtures of $CaH_4(PO_4)_2$, $NaNO_3$ and K_2SO_4 have an accelerating effect. In testing the action of these salts in solutions containing, respectively, 100 parts per million of P_2O_5, NH_3 and K_2O on commercial malt diastase in conjunction with starch paste, it was found that a solution of $CaH_4(PO_4)_2$ increased the diastatic activity of the enzyme from 8 to 16 per cent; that the mixture of equal parts of $CaH_4(PO_4)_2$, and $NaNO_3$ and K_2SO_4 increased the diastatic activity from 6 to 16 per cent while K_2SO_4 alone (100 parts per million) uniformly retarded the enzymatic action and sodium nitrate made little change in the rate of conversion of the starch to reducing sugar. Plants were then grown in bottles containing 100 parts per million of P_2O_5, NH_3, and K_2O or a mixture of equal parts of such solutions and 200 mgm. of starch paste. It was found that the roots of plants growing in these solutions have the power of converting the starch of the solution to sugar. As a rule this diastatic activity was retarded by K_2SO_4 and $CaH_4(PO_4)_2$, but was increased by $NaNO_3$ and the mixtures of the three salts.

SOLUBLE CHITIN.

By CARL L. ALSBERG and C. HEDBLOM.

(From the Laboratory of the U. S. Bureau of Fisheries at Woods Hole and the Department of Biological Chemistry, Harvard Medical School.)

The chitin of *Limulus* resembles other chitins in composition. If treated with 10 per cent hydrochloric acid at room temperature for nine months it acquires the power of gelatinizing and forming colloidal solutions. While the untreated decalcified chitin is colored by iodin, the colloidal solutions have lost this power. These solutions are irreversible, for, when evaporated to dryness, the residue cannot easily be put into solution again. Soluble chitin does not reduce Fehling's solution. On analysis its oxygen content, as compared with the original chitin, is much increased. It dialyzes exceedingly slowly and shows very peculiar osmotic properties demanding further investigation. The possibility of preparing chitin in soluble form will make possible the study of intermediate decomposition products and thus further the study of this constitution.

STUDIES IN COMPARATIVE PHYSIOLOGICAL CHEMISTRY.

By WALTER JONES and JAIME de ANGULO.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

The livers of the adult dog and pig represent two types of nuclein metabolism. In both species, the nucleic acid of the gland is decomposed by the nuclease present, giving rise to guanine and adenine. In the dog's liver the guanine thus formed is promptly converted into xanthine while the adenine does not suffer any analogous change. On the contrary the pig's liver exerts no action on guanine but converts the adenine successively into hypoxanthine, xanthine and uric acid. As the pig embryo develops, the nuclein ferments of the liver appear in the order adenase, xantho-oxidase, guanase (the latter never appearing) and the ferment formation is completed either at birth or shortly after. On the other hand adenase which is present at an early stage of embryonic life in the pig's liver is never present in the

dog's liver. In the case of the dog's liver all nuclein fermentation begins after birth. The livers of several newly born dogs were made into an emulsion with water and to portions of the aqueous extract were added guanine and adenine. The materials were submitted to digestion at the body temperature for the greater part of a year, yet at the end of this time the guanine and adenine could be recovered unchanged.

TESTS FOR PUS AND BLOOD.

By J. H. KASTLE and NORMAN ROBERTS.

(From the Division of Chemistry, Hygienic Laboratory, U. S. Public Health and Marine-Hospital Service, Washington, D. C.)

In 1899, Carriere,[1] in a communication on the presence of indirect-oxidases (peroxidases) in the normal and pathological liquids of man, called attention to the frequent occurrence of peroxidases in the urine of diseased persons and to the absence of such oxygen carriers in the urine of sane, normal persons. By means of very sensitive peroxidase reagents[2] we have examined the urine of 175 persons for peroxidases, with the result that no evidence of the presence of such substances was obtained in strictly normal urines. While positive tests for the peroxidases are frequently obtained with diseased and abnormal urines, their presence does not seem to be characteristic of any particular disease, except such as involve an active inflammation of the genito-urinary tract. The presence of peroxidases in the urine, however, appears to be constant for active inflammations of this character, and generally speaking, is indicative of the presence of pus in the urine. The peroxidase reagents employed by us have been found to be very delicate reagents for the detection of pus and by means of the paraphenylenediamine peroxidase reagent, leucocytes from whatever source are stained blue.

Our investigations on the use of phenolphthalin as a reagent for the detection of blood have been continued.[3] It has been found

[1] *Compt. rend. soc. de biol.*, li, p. 569.

[2] Kastle and Porch: *Journ. Biol. Chem.*, iv, pp. 301,320; 1908.

[3] See Bulletin no. 31, Variations in the Peroxidase Activity of the Blood in Health and Disease, by J. H. Kastle and H. L. Amoss, Hyg. Lab. U. S. Pub. Health and Mar.-Hosp. Service., Wash.

possible to detect 1 part of blood in 80 million parts of water, from which it would seem that the hemoglobin in 200 red cells is sufficient to oxidize measurable amounts of phenolphthalin to phenolphthalein in 5 minutes at ordinary temperature. Blood stains 20 months old also gave beautiful tests with the phenolphthalin reagent.

THE ELIMINATION OF BARIUM.

By GUSTAVE M. MEYER.

(From the Laboratory of Biological Chemistry of Columbia University at the College of Physicians and Surgeons, New York.)

Experiments were carried out on dogs, to determine whether or not barium is eliminated in the urine after its introduction into the animal body. The barium, as the bromide was introduced in varying amounts subcutaneously and *per os*. The methods of analysis employed permitted the detection of 0.2 mg. of barium when added to 360 cc. of urine.

The results indicate that barium is not eliminated in the urine in such amounts as may be detected by chemical means when introduced in fairly large doses *subcutaneously* in dogs. When barium bromid is given *per os* in fairly large amounts only traces of barium appear in the urine.

THE TIME RELATIONS IN THE ELIMINATION OF PROTEINS.

By CHARLES G. L. WOLF.

(From the Department of Chemistry, Cornell University Medical College, New York City.)

In order to determine the rate of deamidation of protein nitrogen, the formation of urea, the oxidation of sulfur to sulfuric acid, and the ratio of carbon to nitrogen and to sulfur, after digestion, an individua was placed on a constant diet, low in nitrogen, for 35 days, and on certain days, meat, plasmon, gelatin, uncoagulated egg-albumin, alanin, cystin, ammonium chlorid, ammonium citrate, urea, asparagin, pancreatic digested egg-ablumin, were superimposed for the original diet at breakfast. For 16 hours, hourly thereafter, the urine was collected, and in each sample the total

carbon nitrogen and sulfur, the ammonia, amid, and urea nitrogen, the total sulfate and neutral sulfur were determined in each experiment, From these data conclusions were drawn regarding the course of the nitrogen and sulfur catabolism after the intake of protein.

STUDIES OF THE INFLUENCE OF VARIOUS DIETARY CONDITIONS ON PHYSIOLOGICAL RESISTANCE.

I. THE INFLUENCE OF DIFFERENT PROPORTIONS OF PROTEIN IN THE FOOD ON RESISTANCE TO THE TOXICITY OF RICIN AND ON RECUPERATION FROM HEMORRHAGE.[1]

By NELLIS B. FOSTER.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Most of the experiments were conducted on pairs of dogs, each pair being kept under identical conditions, so far as they could be controlled, except with respect to the food. One animal of each pair was given *liberal* amounts of protein in the daily diet, the other received *barely sufficient* protein to provide for the necessary nitrogenous metabolism, the remaining ingredients of the food for each animal being uniformly equal to the daily amounts ordinarily given per kilo, in this laboratory, to perfectly healthy dogs. No attempt was made to maintain equal caloric values in the diets. Each animal was given a diet of hashed lean meat, cracker mea and lard, the meat being gradually increased or diminished to a high or low plane, according to the plan in each case and before the particular pathological condition was induced.

[1] This study was begun during the summer of 1905, at Dr. Gies' suggestion and has been carried forward from time to time under his direction and with the aid of a grant from the Rockefeller Institute. I am also indebted to Dr. Flexner for important suggestions.

The work has been frequently interrupted by researches in other directions and has been beset by unusual experimental difficulties. Although planned to be the first of a series of investigations, in point of publication it is the second from this laboratory on the general subject stated above. See Dissertation by Welker, Columbia University, 1908. It is Dr. Gies' intention to continue investigation along these lines.

The dogs were under daily observation for periods of at least two months and, in several cases, four months before inauguration of the pathological phase.

In testing the physiological resistance of the animals, two methods were used: (1) hypodermic injection of ricin and (2) blood-letting.

The accompanying table presents a summary of the results.

GENERAL SUMMARY.

Totals (Experiments I–XII)	High plane of protein nutrition—6 dogs	Survived	2
		Died	4
	Low plane of protein nutrition—5 dogs	Survived	2
		Died	3
Ricin Experiments	High plane of protein nutrition—3 dogs	Survived	0
		Died	3
	Low plane of protein nutrition—2 dogs	Survived	1
		Died	1
Hemorrhage Experiments	High plane of protein nutrition—3 dogs	Survived	2
		Died	1
	Low plane of protein nutrition—3 dogs	Survived	2
		Died	1

ON THE PRESERVATION OF FECES.

By PAUL E. HOWE, T. A. RUTHERFORD and P. B. HAWK.

(*From the Laboratory of Physiological Chemistry of the Department of Animal Husbandry of the University of Illinois.*)

The feces was collected in the ordinary friction-top paint pail and during the preservation period kept in a "cold room". The feces was analyzed for moisture and nitrogen immediately after defecation and at short intervals thereafter. A typical analysis follows:

	FIRST ANALYSIS.	2 DAYS.	4 DAYS.	8 DAYS.
Nitrogen (Moisture-free)	1.582	1.597	1.622	1.618
Moisture	74.85	74.62	74.40	74.93

CONCLUSIONS.

I. The method of feces collection and preservation which involves the use of friction-top pails is very satisfactory for the following reasons:

1. It permits of the analysis of the *fresh* feces.
2. It prevents loss of moisture.
3. It maintains the nitrogen content practically unaltered for at least *twenty days* and frequently for a much longer period.
4. It eliminates all loss of material since the feces is not transferred to any other receptacle before it is thoroughly mixed for analysis.

II. It is preferable to make the analysis on the *fresh* feces, since this procedure does away with all drying processes and hence eliminates the loss of nitrogen which invariably accompanies such drying.

A STUDY OF NUCLEOPROTEIN FROM THE GASTRIC MUCOSA.

BY ARCHIBALD E. OLPP. (By invitation).

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Acid-precipitable nucleoprotein has been extracted from the gastric mucosa with dilute alkaline solution. The purified product appears to be free from mucin. The results of a special study of its properties will shortly be published in detail. It is planned to investigate also the possible relation between the gastric nucleoprotein and pepsinogen.

THE ANALYSIS OF THE LEUCIN FRACTION IN PROTEIN HYDROLYSIS.

BY D. D. VAN SLYKE AND P. A. LEVENE.

(From the Rockefeller Institute for Medical Research, New York.)

In the isolation of amino-acids from proteolytic products, leucin, isoleucin, and valin are almost invariably obtained as a hitherto inseparable mixture. These acids, and likewise their copper salts, form isomorphous mixtures inseparable by crys-

tallization, and their esters have so nearly the same boiling points that they distil as one fraction. Consequently most investigators have not attempted a separation, but have reported the entire mass as "leucin."

We have succeeded in separating readily and quantitatively the leucin isomers from the valin. The method rests on the fact that if a molecular lead acetate solution is added to an ammoniacal solution of the mixture, the leucins are precipitated as analytically pure $Pb(C_6H_{12}O_2N)_2$. The ammoniacal solution is prepared by suspending the acids in 7 parts of hot water, and adding 1 to 2 parts of aqueous ammonia. An estimate of the proportion of leucin is calculated from the carbon content of the mixture, and 20 per cent excess of lead acetate used. More may precipitate valin. The latter is obtained analytically pure by freeing the filtrate from lead with hydrogen sulphide, evaporating to dryness, and washing with absolute alcohol. A slight amount of valin dissolves, but is regained by evaporating the filtrate.

Levene and Jacobs[1] showed that leucin and isoleucin can be readily separated when freed from valin. Consequently the systematic isolation of all three is now rendered comparatively easy This is of importance, not only for protein analysis, but also for the preparation of pure, optically active valin and isoleucin, a task hitherto extremely difficult.

FURTHER STUDIES OF PROTEIN COMPOUNDS.

By WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

The following new products have lately been subjected to study from the special standpoints of preparation and properties: Copper, silver, trimethylamin, guanidin, and histon compounds of mucoid; copper, silver and histon compounds of nucleoprotein; potassium, calcium and copper compounds of alkali albuminate.

Pure products of striking osmotic power have been prepared. Some of them, including silver compounds (after removal of

[1] Levene and Jacobs: *Biochem. Zeitschr.*, ix, p. 231, 1908.

impurities by dialysis), show in aqueous solution remarkable resistance to precipitation by alcohol and by alcohol-ether. Some of the *pure*, moist products are quite soluble in 90 per cent alcohol.

The study is progressing with the help of Messrs. W. H. Eddy, M. G. Herzfeld, H. E. Buchbinder, O. C. Pickhardt and E. W. Baker.

The inorganic composition of the blood of Limulus. By A. B. MACALLUM.

On the function of the parathyroid gland. By CARL VOEGTLIN and W. G. MACCALLUM.

A study of the metabolism in Myositis ossificans. By RALPH W. WEBSTER.

On the nature of copper-protein compounds, and the effect of copper upon digestion. By HARVEY W. WILEY and J. A. HERMAN SCHREIBER.

EDITORIAL ANNOUNCEMENT.

The Editorial Board of the *Journal of Biological Chemistry* announce with regret the withdrawal of Prof. John J. Abel from the active editorial management of the *Journal.* Professor Abel has deemed it necessary to take this step in order to secure time and energy to actively conduct the newly projected *Journal of Pharmacology and Experimental Therapeutics.*

In his letter of resignation Professor Abel says: "Although my editorial labors must now be applied elsewhere, the *Journal of Biological Chemistry* will always have my hearty interest and coöperation." Professor Abel will, accordingly, remain connected with the *Journal* as a member of the Board of Associate Editors, in which capacity he will continue to sustain the aims for which it was founded. While the *Journal* must thus in part relinquish its claims on Professor Abel's time and energy, it is surely cause for congratulation that closely affiliated and highly important interests in biological science will be correspondingly advanced.

THE SPONTANEOUS OXIDATION OF THE SUGARS.[1]

By A. P. MATHEWS.

(From the Laboratory of Biochemistry and Pharmacology, University of Chicago.)

(Received for publication, January 24, 1909.)

How does it happen that the proteids, carbohydrates and fats which ordinarily are oxidized by atmospheric oxygen at a very slow, or almost imperceptible rate, combine with oxygen with great rapidity in living matter?

To answer this question I made the hypothesis that the total consumption of oxygen by the protoplasmic mixture is the sum of the consumptions of the various substances composing that mixture. If, therefore, we wish to know why sugar or any other substance burns rapidly in the living mixture, one way of finding out would be to discover what had to be done to that substance to make it burn rapidly outside of the cell. We might then infer that a similar thing was done in living matter and proceed to look for evidence that such a process actually took place. I have, hence, been studying the spontaneous oxidation of various cell constituents *in vitro* and I began with the sugars.

If glucose is dissolved in water, the solution sterilized, and left exposed to air or oxygen at room temperature the oxidation goes on at so slow a rate as to be practically imperceptible for long periods. Yet this same glucose burns rapidly in living matter. There are two ways in which with present chemical conceptions we may picture the mechanism of this acceleration: the active mass of the oxygen or of the sugar molecules, or of both is actually increased, or the speed of the reaction is accelerated by the formation of intermediate compounds. It may be

[1] The experimental part of this paper was carried on at the Marine Biological Laboratory, Wood's Hole, during the summer of 1907. After the completion of this paper, that by Bunzel and the writer was begun. In the interim also, Nef's paper appeared which anticipated a portion of the conclusions of this paper. (Nef: *Ann. d. Chem.* cccvlii, p. 214, 1908.)

that the proportion of molecules of oxygen or sugar which are in an active state, that is capable of combining, is, ordinarily very small, whereas in living matter the proportion of active molecules is increased. Nearly all of our hypotheses of respiration and oxidation have been directed hitherto to the oxygen. It has been generally assumed that the oxygen of air is relatively inactive and that it is activated in living matter by conversion into atomic oxygen, ozone or peroxide so that the acceleration of oxidation is due to this factor. The results of the following investigation indicate clearly that the more important preliminary change resulting in oxidation is in the reducing substance, the active mass of this being potentially or actually increased so that not only does it possess a greater speed of oxidation, but it becomes capable also of all those other chemical transformations visible in growth, differentiation and metabolism. From this point of view respiration will be seen to be only an incidental or special case of the general process of metabolism and growth.

I. THE SPONTANEOUS OXIDATION OF THE SUGARS.

The observations of McGuigan,[1] Bunzel[2] and the author[3] showed that the different sugars levulose, galactose, glucose, maltose and lactose oxidized in an acid medium in the presence of copper salts, i.e., cupric acetate, at very different velocities, levulose burning the most rapidly and the other sugars following in the order named to lactose which, with the exception of cane sugar, burned least rapidly. Furthermore we found that the degree of acidity, i.e., the number of hydrogen ions, greatly influenced the rate, the rate diminishing with an increase of hydrogens. The explanation offered by us was that the sugars reacted like weak acids and that their dissociation, which was a necessary preliminary to oxidation, was reduced by the presence of hydrogen ions. This explanation was confirmed by subsequent studies by Bunzel and the writer.[4] The hydrogen ions, also, reduced the number of oxygen ions. The reduction in speed by acids was

[1] McGuigan: *Amer. Journ. of Physiol.*, xix, p. 175, 1907.
[2] Bunzel: *Ibid.*, xxi, p. 23, 1908.
[3] Mathews and McGuigan: *Ibid.*, xix, p. 199, 1907.
[4] Bunzel and Mathews: *Journ. of the Amer. Chem. Soc.*, xxxi, 1909.

due to both factors and there is no doubt from the results of the present investigation, as well as those of the first, of the importance of the ionization of the sugar as a prerequisite of oxidation.

The oxidation of sugars by air in an alkaline solution has been studied by Framm[1] and the greater speed of oxidation of levulose observed. But the relative velocities of oxidation of the different sugars in alkaline solutions have not been studied, and the influence on velocity of varying concentrations of alkali has not been determined, nor have the reactions been explained.

The method employed was to bring 2 grams of a monosaccharide or 4 grams of a disaccharide into a 400 cc. flask which was provided with a ground glass stopper carrying a mercury manometer and a glass cock; then to add 50 cc. of alkali; and to shake the contents in a shaking machine so violently as to secure a thorough mingling of the air and solution. The solution under these circumstances absorbs the oxygen in the flask, and the amount absorbed was determined by the negative pressure in the flask as measured by the manometer. The barometer and the thermometer were read at the same time. All experiments were carried on at room temperature which varied a good deal, but which was ordinarily about 23–25° C. I had no means of shaking the flasks at constant temperature. The variations in temperature introduce certain irregularities into the results, but do not materially affect the main results.

a. The rate of oxidation of the different sugars at constant alkalinity.

TABLE I.

Comparative absorption of oxygen by levulose, galactose, and glucose, 0.4 N KOH.

Time shaken in minutes.	ABSORPTION OF OXYGEN IN MM. Hg.				
	Levulose.	Galactose.	Glucose.	Temp. °C.	Barometer.
30	27	5	6	24.8	762
60	52	13	14	24.9	762
90	74	22	23	24.9	762
110	88	30	30	24.8	762
170	118	56	53	24.7	762
210	132	70	63	24.7	762

[1] Framm: *Arch. f. d. ges. Physiol.*, lxiv, p. 575, 1896.

TABLE II.

Comparison of levulose, glucose and maltose, 0.8 N KOH.

Time minutes.	ABSORPTION OF OXYGEN IN MM. Hg.				
	Levulose.	Glucose.	Maltose.	Temp. °C.	Barometer.
30	33	13	10	24.9	760
60	62	21	21	24.9	760
90	83	35	30	24.9	760
110	95	40	38	24.9	760
210	130	80	79	24.9	759

TABLE III.

Comparison of rate of oxidation of lactose and glucose in N KOH.

Time shaken in minutes.	ABSORPTION OF OXYGEN IN MM. Hg.			
	Glucose.	Lactose.	Temp. °C.	Barometer.
15	5.5	6	22.7	757
32	11.5	12	22.8	757
47	17	18	23.1	757
90	35	36	23.6	757
157	66	65	25	757
244	115	104	26.5	757
320	133	123	27.5	757

The experiments recorded in Tables I, II and III and in the curves of Figs. I and II show, as was already well known, that all these sugars oxidize easily spontaneously in an alkaline solution, and that the levulose oxidizes more rapidly than the others. The differences between the rest are not marked, but galactose oxidizes slightly faster than glucose. Glucose, maltose and lactose oxidize with approximately the same speed and this behavior in alkaline solution is in marked contrast with their comparative velocities in acid solution,[1] in which a great difference exists between the speed of oxidation of glucose and maltose and a considerable difference in the velocity of oxidation of lactose and maltose. In other words, the velocity in alkaline solution is much greater than in acid and the differences between the sugars largely disappear in the former. So far, however, as differences

[1] Mathews and McGuigan: *loc. cit.*

exist in the alkaline oxidations they are in the same direction as the acid oxidations. In both cases levulose goes fastest. The actual speed of oxidation of the levulose by air in alkaline solution is considerable. In 0.5 N alkali three-fifths of the total oxygen is removed from the air of the flask in two hours at room temperature and with the levulose in the concentration of about 4 per cent. If we suppose that four atoms of oxygen combine with one atom of sugar, an assumption which is of course not strictly justified, this would account for about 0.2 gram of levulose oxidized in this time. The reaction is too complex to permit of any accurate estimation of the amount of levulose actually destroyed in that interval.

The comparative rate of oxidation may be made more obvious by the use of the velocity constants given by an equation of the first order. The change in concentration of the oxygen is so great and the change in concentration of the sugar is so little, owing to the large amount of sugar taken and to the fact that the degradation products of the levulose are also reducing agents, that for the sake of comparison an equation involving the oxygen only may be used, although it does not represent the real course of the reaction. Also owing to the change in temperature in some of the experiments and the unknown real change in the sugar, the constants in general increase in the course of the experiment. They serve, however, to give a numerical comparison of oxidation velocity. The constants calculated from the tables given are as follows:

$$\frac{1}{t} \log \frac{A}{A - x} = K$$

Time minutes.	Levulose.	Glucose.
30	0.00272	0.00056
60	0.00291	0.00067
90	0.00294	0.00076
110	0.00322	0.00083
170	0.00352	0.00104
210	0.00374	0.00105
Mean K	0.00317	0.00082

In other words, the mean velocity of the oxidation of levulose in 0.4 N potassium hydrate is a little less than four times that of

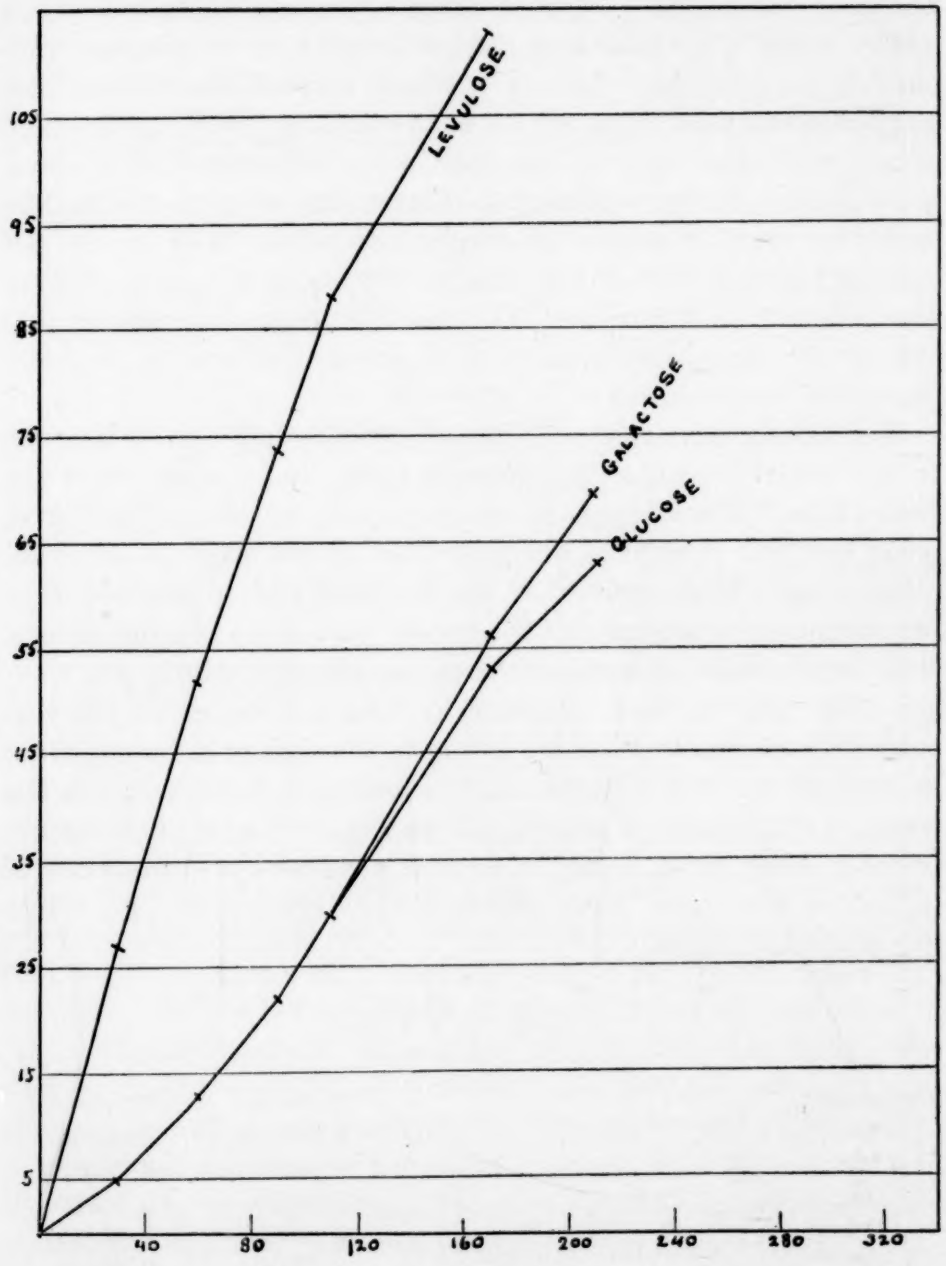


Fig. I. Curves showing the rate of absorption of oxygen by levulose, glucose and galactose when dissolved in 0.4 N potassium hydroxide. On the abscissa the figures represent time in minutes; the ordinates represent mm. of negative pressure in the flasks due to oxygen absorption.

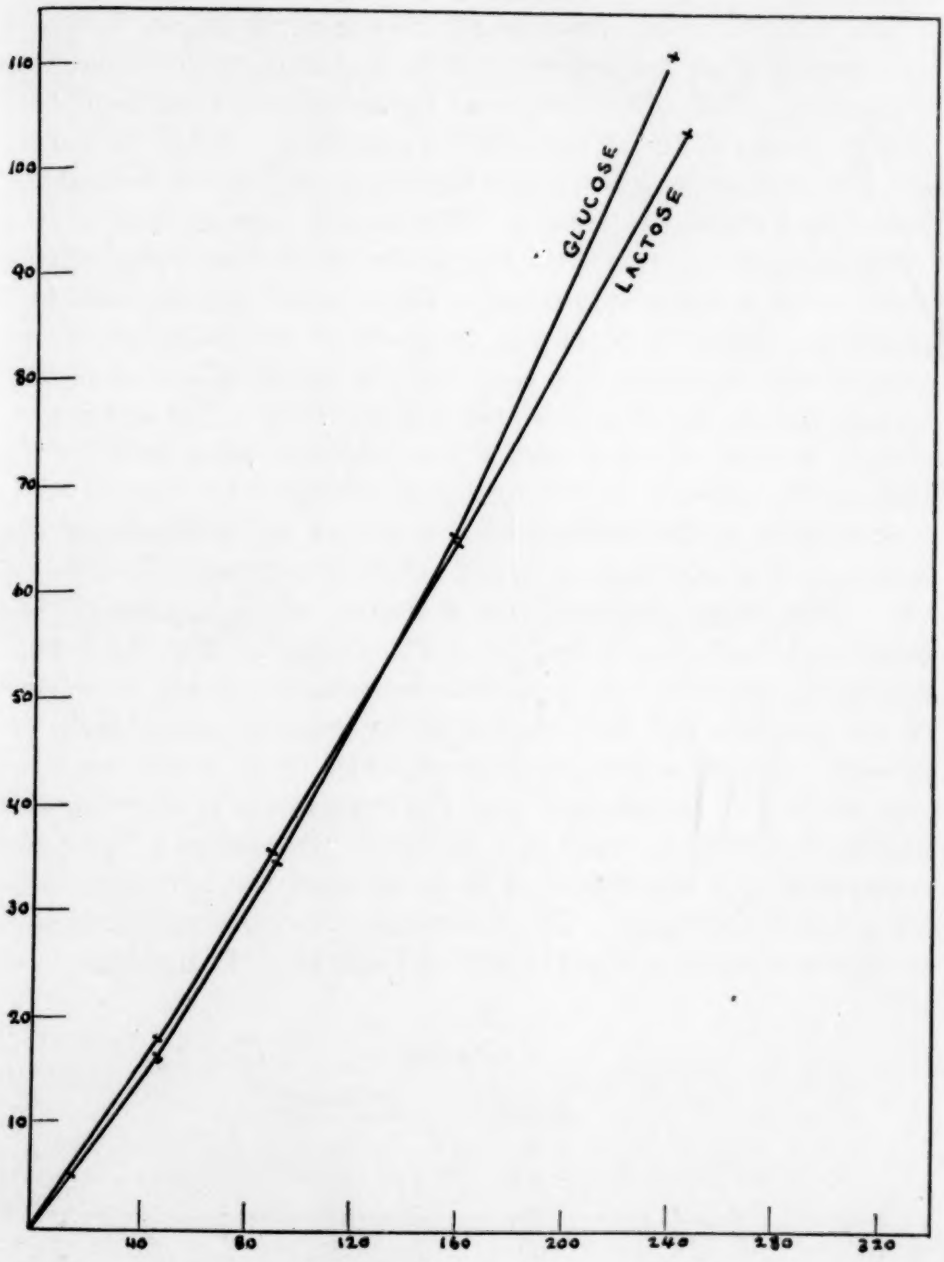


Fig. II. Curves showing the rate of absorption of oxygen by glucose and lactose when dissolved in N potassium hydroxide. Ordinates and abscissas as in Fig. I.

glucose. The other sugars have about the same velocity as glucose.

The velocity of oxidation of all the sugars undergoes a steady acceleration from the beginning of the reaction and for some time thereafter. This will be observed by an inspection of the curves in Figs. I and II and of the velocity constants. All of the curves but levulose are for a time after the beginning convex toward the base line instead of concave. This shows that in spite of the diminution in the concentration of the oxygen the speed of the reaction is actually increasing. There were two explanations possible. Either some of the products of the oxidation acted catalytically to hasten the reactions, or the alkali increased the concentration of the active reducing particles. The latter possibility proved to be correct. The addition of several centimeters of a partially oxidized solution of maltose to a fresh solution did not in the least affect the rate of the oxidation of the latter, as it would have done had catalytic substances been present. The other possibility is therefore what happens: The alkali as is well known decomposes the sugar so that the actual number of active reducing particles is increased during the course of the reaction and the velocity of the reaction accordingly increased. To show how previous exposure to an alkali increases the velocity of oxidation, I tried the experiment of allowing two grams of glucose to stand in $\frac{N}{2}$ sodium hydroxide in a hydrogen atmosphere for two days. I then replaced the hydrogen with air and shook as usual. The absorption of oxygen was very rapid as shown in curve 1, Fig. III and by Table IV. Temperature 25–26°.

TABLE IV.

Time in minutes.	Absorption in mm. Hg.
10	22
30	43
50	61
60	67
80	80
90	88
115	100
130	111
240	140
360	152

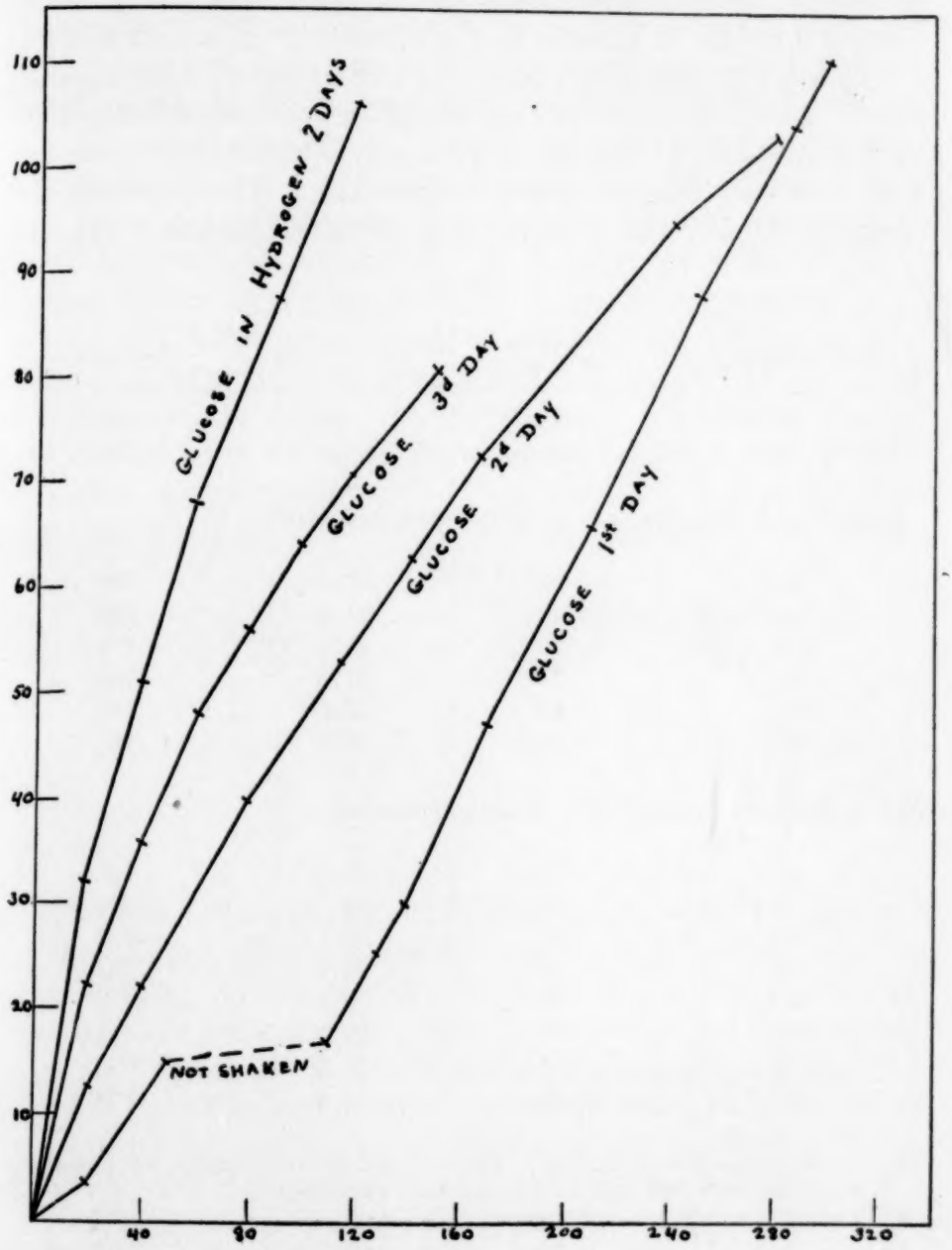


Fig. III. Curves showing the rate of absorption of oxygen by glucose when dissolved in $\frac{N}{2}$ sodium hydroxide and shaken on successive days, or exposed to hydrogen gas for 2 days before shaking. Coördinates same as Fig. I.

The absorption in this case was more rapid even than that of levulose. Another experiment of the same kind was tried by placing 2 grams of glucose in $\frac{N}{2}$ sodium hydroxide and shaking it on three successive days renewing the air before shaking each time. The absorption was more rapid on the second day than on the first, and on the third day it was as rapid as levulose and the curve was concave toward the base line. This experiment is shown in Table V and graphically in the curves plotted in Fig. III.

TABLE V.

Time in minutes.	Absorption mm. Hg.	Temperature.	Barometer.
20	3.5	26	746
50	13	26	746
Interval of 60 minutes not shaken; 4 mm. absorbed.			
145	27	27.1	746
175	46	27.1	746
230	73	27.6	746
270	93	27.6	746
325	116	27.6	746
345	122	27.6	746
Not shaken for 17 hours. Air renewed second day.			
30	19.5	23.5	750
60	28	24	750
80	35	24.7	750
144	57	26.6	752
180	68	27.1	752
216	76	27.5	752
236	81	28.1	752
276	88	28.5	754
Interval of 17 hours not shaken. Flasks stood closed at room termperature. The air then renewed and flasks shaken. Third day.			
10	10	26	759
30	26.5	26.5	759
60	45	27.5	759
90	58	27.2	759
120	68	27.2	759
150	78.5	27.2	759

These experiments show that under the action of the alkali the glucose molecule is broken up into a larger number of actively reducing molecules. At the same time there is produced a considerable quantity of acid even in the absence of air. We may infer therefore that in the rearrangement an intramolecular oxidation and reduction takes place. I made a couple of experiments to follow the rate of the formation of acid from sugars by alkalies in the absence of oxygen and from one of these experiments in Table VI it will be seen that the curve of the formation of acid is similar to that of the acceleration of the reducing process.

Six small flasks were completely filled with a 4 per cent solution of glucose and then inverted over mercury so that no air had access to the flasks. At varying intervals of time the contents of the flasks were titrated against $\frac{N}{2}$ sulphuric acid, phenolphthalein being the indicator. The flasks stood at room temperature, about 26° C.

TABLE VI

Time in hours of exposure to the alkali.	Cc. of $\frac{N}{2}$ sulphuric acid required to neutralize 10 cc. of alkali-sugar mixture at end of time intervals.
0	10.03
5	10.00
23	9.60
47	8.82
69	7.92
93	6.76

It will be noticed that there is a loss of alkalinity of 4 per cent of the original alkalinity the first 23 hours; of 7.8 per cent the second 24 hours; of 9 per cent the third, and of 11.6 per cent the fourth. The acids are produced at a constantly increasing speed. This shows that the acids are formed secondarily, and the first effect of the alkali is to produce compounds easily oxidized, which secondarily become acids, presumably by intramolecular oxidation or by the reduction of some of the reaction products.

b. The influence of the concentration of the alkali on the velocity of oxidation.

Corresponding with the conclusions of those who have studied the oxidation of pyrogallic acid,[1] it was found that there is an

[1] Weyl and Goth: *Ber. d. deutsch. chem. Gesellsch.*, xiv, p. 2659, 1883; Berthelot: *Compt. rend. de l'acad. d. sci.*, cxxvi, pp. 1066 and 1459.

optimum concentration of alkali. The velocity is greatest in N to 2N solutions and falls off in either direction from this optimum.

TABLE VII A.

Oxidation of levulose in different concentration of NaOH.

Time shaken minutes.	ABSORPTION IN MM. Hg AT VARIOUS CONCENTRATIONS NaOH.					
	N.	$\frac{N}{10}$.	$\frac{N}{100}$.	$\frac{N}{1000}$.	Temperature.	Barometer.
20	25	2.5	0.5	0	24.7	762
40	47	6	1.5	0	25.	762
60	64	9	1.5	0	25.8	762
83		14	2.5	0.5	26.1	762
100		18	2.8	0.5	26.6	762
128		21	0.3	0.8	27.1	762
255		47	3.9	1.0	28.2	762

TABLE VII B.

Time shaken minutes.	4N NaOH.	2N NaOH.	N NaOH.	$\frac{N}{5}$ NaOH.	$\frac{N}{10}$ NaOH.	Temperature.	Barometer.
20	3.5	13	23	9	5	25.4	764
40	9	23.5	45.5	20	8.5	25.6	764
60	14.5	36	62	29	11	26	764
90	26	55	86	47	21	26.1	764
110	33	66	99	60	27	26.5	764
142	broken	80	113	74	34	26.5	764
solution at end	very brown	brown	lt. brown	colorless	colorless		

TABLE XI.

Oxidation of glucose in different concentrations of alkali.

Time in minutes.	O_2 ABSORPTION IN MM. Hg AT VARIOUS CONCENTRATIONS NaOH.					
	$\frac{N}{10}$.	$\frac{N}{5}$.	N.	2N.	4 N.	Temperature.
20	0	0	6	9.5	4	25
60	1.5	4	20	29	16.5	25.6
95	4	10	38	47	27	26.5
215	6.5	28	93	103	60	26.5
275	10	41	115	119	72	27.2
340	13.5	56	colorless	lt. brown	86	27.1

TABLE XII.

Oxidation of galactose in different concentrations NaOH.

Time in minutes.	O_2 ABSORPTION IN MM. Hg.				
	$\frac{N}{2}$.	N.	2N.	4N.	Temperature.
30	17	19	16	8	25.8
60	28	35	29	14	26
120	60	66	59	31	27
color of solution at end	colorless	brown	dark brown	dark brown	

TABLE XIII.

Oxidation of lactose in different concentrations of NaOH.

Time in minutes.	ABSORPTION IN MM. Hg.					
	$\frac{N}{10}$.	$\frac{N}{2}$.	N.	2N.	4N.	Temperature.
138	3.5	55	77	82	17.5	26.7
180	4	70	96	100	cracked	27.1
240	7.5	90	115	115	cracked	27.1
513	20	119	140	139	cracked	27.1

TABLE XIV.

Comparison of oxidation of lactose and glucose.

Time in minutes.	ABSORPTION IN MM. Hg.					Temperature.
	Lactose $\frac{N}{2}$ NaOH.	Lactose N NaOH.	Lactose 2N NaOH.	Lactose 4N NaOH.	Glucose 2N NaOH.	
30	5	9	11	7	9	24
80	17	29	41	19	35	25
100	21	38	49	23	40	25.3
257	64	94	99	58	103	27.8
288	71	103	106	65	109	28.1
343	87	117	117	77	122	28.1

TABLE XV.

Oxidation of glucose in 1 per cent solution in different concentrations NaOH.

Time in minutes.	ABSORPTION IN MM. Hg.					Temperature.
	$\frac{N}{10}$.	$\frac{N}{5}$.	$\frac{N}{2}$.	N.	2N.	
22	1	2	3	2	2	24.5
40	3	4	5.5	6	5	24.8
78	4	5.5	8.5	11	11	25.1
120	7	10	14	18	19	25.5
360	22	31	49	55	62	26

TABLE VIII.

Velocity constants of levulose oxidation $\frac{1}{t} \log \frac{A}{A-x} = K.$

Time in minutes.	CONCENTRATION OF ALKALI.					
	$\frac{N}{10}$.	$\frac{N}{5}$.	$\frac{2N}{5}$.	N.	2N.	4N.
18		0.00118		0.00380	0.00176	0.00062
30			0.00271			
40	0.00060	0.00147		0.00394	0.00171	0.00071
60	0.000523	0.00147	0.00289	0.00361	0.00181	0.00072
90	0.000721	0.00175	0.00305	0.00389	0.00206	0.00089
120	0.000756	0.00196	0.00322	0.00397	0.00216	0.00103
140	0.000774	0.00188		0.00391	0.00215	
170			0.00352			
Mean *K*	0.000675	0.00162	0.00308	0.00385	0.00194	0.00079

TABLE IX.

Velocity constants of glucose oxidation.

Time in minutes.	CONCENTRATION OF ALKALI.					
	$\frac{N}{10}$.	$\frac{N}{5}$.	$\frac{2N}{5}$.	N.	2N.	4N.
60	0.000069	0.00028	0.00067	0.00098	0.00147	0.00080
93	0.000089	0.00037	0.00076	0.00130	0.00165	0.000894
215	0.000085	0.00046	0.00083	0.00181	0.00258	0.000977
275	0.000103	0.00053	0.00104	0.00206	0.00221	0.000962
341	0.000114	0.00059				0.00100
Mean *K*	0.000093	0.00044	0.00082	0.00154	0.00198	0.00093

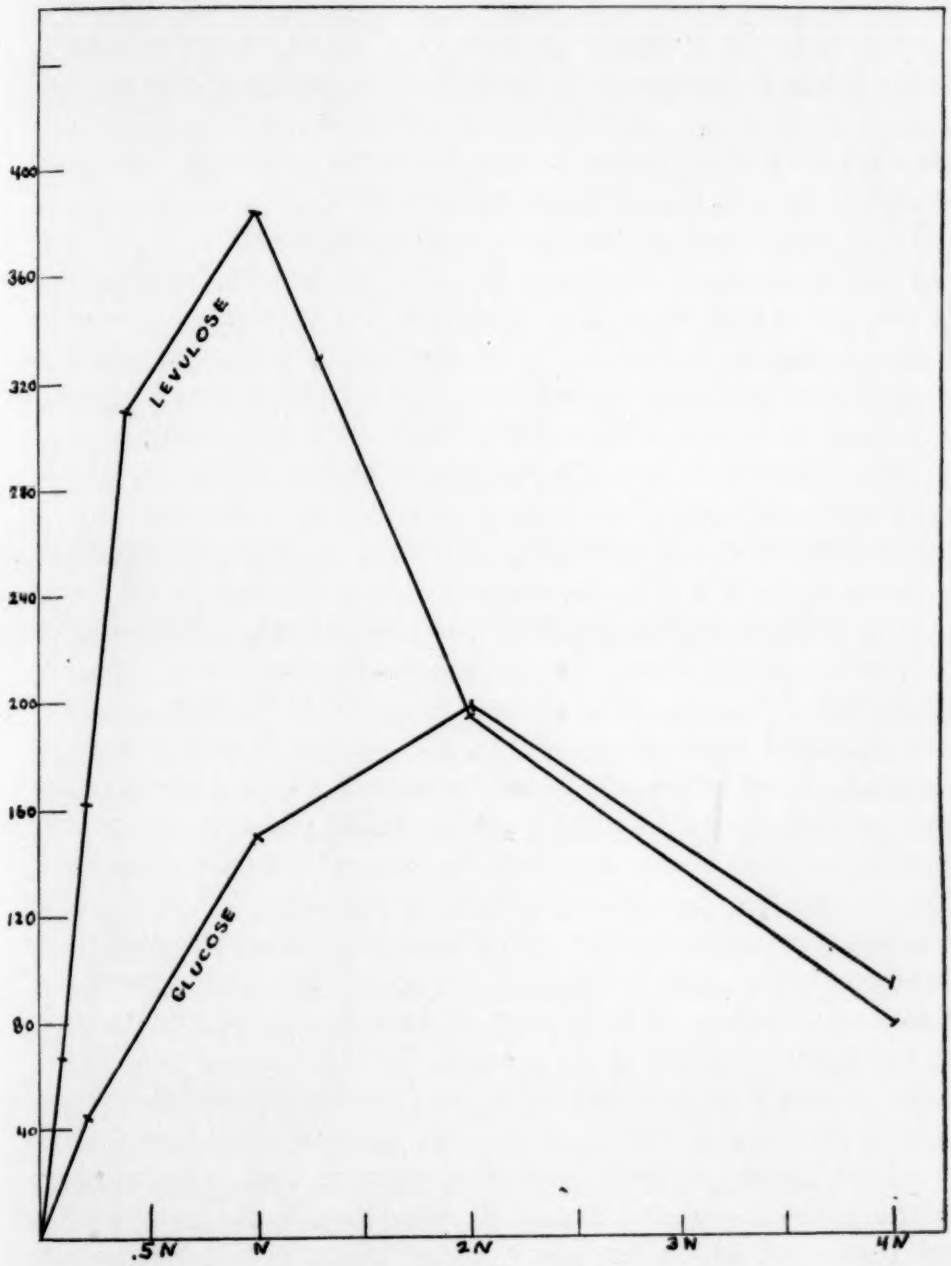


Fig. IV. Curves showing the influence of alkali on the velocity constants of the oxidation of glucose and levulose. Abscissa represents concentrations of sodium hydroxide; ordinate figures are mean velocity constants from Tables VIII and IX multiplied by 100,000.

According to the Tables VII–XV and the curve in Fig. IV the optimum concentration of alkali is seen to be about N sodium hydroxide for levulose and galactose and 2N for the other sugars. This optimum appears to be largely independent of the concentration of the sugar, as is shown by Table XV, in which the concentration of the glucose is only one-fourth that in the other experiments, but the optimum remains at 2N.

The explanation of these results is as follows:

The sugars react essentially like very weak acids,[1] that is, one of the hydrogens dissociates as an ion. The dissociation constant of glucose is 6×10^{-13}. It may be that this hydrogen is that of the hydroxyl immediately behind the aldehyde group, or it may be that a preliminary addition of water similar to that of chloral hydrate takes place in the aldehyde group and one of the hydrogens then dissociates or possibly as Nef suggests it is not always the same hydrogen. However formed, the free acid, i.e., the sugar, is stable and scarcely dissociated, but in the presence of alkali a small amount of a salt is formed. This sodium glucose compound dissociates and there is thus produced a glucose anion with a free negative charge. As a result of the presence of this partially unbalanced charge, the electrical equilibrium of the anion is disturbed, the bonds between the atoms are loosened and a rearrangement of the molecule takes place, in the course of which several new compounds are formed. What these compounds are, Nef[2] has more particularly studied. These unstable reaction products undergo oxidations, condensations or further decompositions giving rise to the various end products of the action of alkalies. The process of dissociation of the sugar is hastened by the addition of more alkali, if the concentration of the latter is small because the hydrolysis of the sodium glucoseate is reduced thereby, but is not greatly accelerated if more alkali is added when a good deal is already present. An increase in the concentration of the alkali, therefore, so far as it affects the sugar should hasten the reaction, since it accelerates the decomposition of the sugar as shown by the rapid browning in strong solutions. The actual slowing of the whole reaction by strong alkali must hence, it seems to me, be due to the

[1] Cohen: *Zeitschr. f. physikal. chem.*, xxxvii, p. 69, 1901.

[2] Nef: *Ann. d. Chem.*, cccIvii, p. 214, 1908.

action of the alkali on the oxygen, since, as is well known, the browning does not take place ordinarily if sufficient oxygen is present. Possibly the oxygen ions in the solution by their osmotic pressure counteract to some degree the solution pressure of the oxygen and hence retard the speed of entrance of the oxygen ions. Or possibly the solubility of oxygen in strong alkali is less. The total effect, therefore, of the addition of alkali is at first to hasten the reaction by increasing the dissociation of the sugar more than it retards the activity of the oxygen. Larger quantities, however, slow the reaction, because the sugar dissociation having reached a high figure is increased less than the speed of the solution of the oxygen is diminished.

The foregoing experiments show in connection with those following on cystein and those described by Bunzel and the writer, that the main reason that glucose does not burn rapidly in the air is not due to the fact that oxygen is not present in an active state. The rapid burning of pyrogallic acid, or cystein and other substances in neutral solutions shows that there is no lack of active oxygen in such solutions. The cause of the failure to oxidize must lie hence in the glucose molecule. This must be activated before it will burn. The activation is brought about by a preliminary ionization in the alkaline solutions, this ionization resulting in an upset of electrical equilibrium and a resulting molecular rearrangement, leading to decomposition of the molecule and the oxidation and condensation of the dissociated parts as Nef has more particularly pointed out. It is possible that a similar process may take place in living matter, some substance being present which unites with the glucose molecule and brings about its dissociation. As a consequence the sugar is burned in part, and in part converted into its other metabolic products.

It is clear from this presentation that in the past, two distinct groups of substances have been confused under the name of oxidases: A group which actually hastens oxidation by its action on the oxygen, of which iron is a good example; and a group specific in their affinities which hasten oxidation by acting on the reducing substances and thus increase their reducing power. These substances might possibly be called "reductases," but it must be remembered that they are responsible not only for

the oxidations but for all the other more important metabolic activities of protoplasm, so that they might more properly be called metabolases, since they hasten metabolism.

SUMMARY.

(1) The sugars levulose, galactose, glucose, maltose and lactose oxidize rapidly in the presence of air if their solutions are alkaline, but not if they are neutral or acid. This shows that for oxidation it is necessary to form a salt of the sugar. This salt ionizes and the resulting disturbance of electrical equilibrium in the anion causes its decomposition and easy oxidation.

(2) Of the sugars, levulose oxidizes the most rapidly, while the others oxidize at about the same speed in alkaline solution and about one-fourth as fast as levulose.

(3) All sugars examined, but preëminently all except levulose, show an acceleration of the velocity of oxidation and decomposition as the reaction goes on. This is due to the fact that by the decomposition of each sugar molecule several reducing molecules are produced so that their concentration is increased.

(4) The oxidation is most rapid in N sodium hydroxide in the case of levulose, and about 2N concentration of sodium hydroxide for the other sugars. Stronger alkali than this reduces the rate of oxidation. This is probably due to the fact that the alkali in strong concentration reduces the oxidation velocity of the oxygen more than it accelerates the decomposition of the sugar.

(5) The failure of glucose to oxidize rapidly in the air in neutral solution is not due to the lack of active oxygen, but primarily to the fact that the glucose will not oxidize until it is dissociated. This shows that a failure of living matter to burn glucose is probably not due to the absence of oxidases, properly speaking, but to the probable loss of its power to dissociate the glucose. Under the term of oxidases there have hitherto been confused two classes of substances; one which activates the oxygen; the other the more important class, which activates by dissociation the reducing substances. The latter are specific; the former not.

THE SPONTANEOUS OXIDATION OF CYSTEIN.

By A. P. MATHEWS and SYDNEY WALKER.

(*From the Laboratory of Biochemistry and Pharmacology, University of Chicago.*)

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The oxidation of cystein is of particular interest from the possible connection of the sulphur compounds of the cell with the respiratory process. Heymans and Masoin[1] have shown that the cyanides, which particularly check cell oxidations, unite, in some animals at any rate, with the sulphur, and Heffter[2] has recently published a paper designed to show the importance of sulphur in respiration. It is probable that the sulphur atom is one of the most labile and reactive in the albumin molecule. All these considerations make the oxidation of cystein of interest biologically.

The method of making cystein easily in large amounts was that of Mörner[3] with certain modifications. As this method may be of use to others we give it in full: Ox horns are allowed to dry until the bony core of the horn may be easily slipped out. The hollow horn is then sawed into sections, the sections dried to make them split easier and then split into small pieces. One kilo of the dried horn is placed in a three or four liter flask and extracted two days or longer at room temperature with 10 per cent hydrochloric acid. The acid is then poured away and two liters of 15 per cent hydrochloric acid are added and the flask heated on the steam bath for seven days under a reflux condenser at a temperature of from 90° C. to 92° C. At the end of seven days the contents are filtered, partially decolorized once with charcoal and the deep red liquid is evaporated *in vacuo* in a water bath until a fairly thick syrup of about 700 cc. remains. The syrup is then poured into a large beaker and strong sodium

[1] Heymans and Masoin: *Arch. internat. de Pharmacodyn.*, vii, p. 77, 1896.

[2] Heffter: *Med.-Naturwiss. Arch.*, Berlin, i, pp. 81–104, 1907.

[3] Mörner: *Zeitschr. f. physiol. chem.*, xxviii, p. 595, 1899.

hydrate is carefully added little by little to the solution until the reaction is faintly acid or neutral to litmus. The solution is cooled and then ethyl alcohol added until the solution contains from 50 to 70 per cent by volume of alcohol and allowed to stand at 0° C. or below for two days. The voluminous, brown precipitate consisting of cystin, tyrosin and some impure leucin which may have already begun to appear before the addition of the alcohol is filtered under suction, is brought into a large evaporating dish and extracted several times, five to seven, with a liter of boiling water each time until the residue gives either no tyrosin reaction, or a faint red only with Millon's reagent, but instead a heavy white precipitate. By extraction with boiling water the tyrosin, leucin and some cystin is dissolved, but since tyrosin is very much more soluble in boiling water than cystin, the great bulk of the cystin remains behind while the tyrosin is removed. Most of the leucin goes into the first extract. The coloring matter also goes into solution. The crude cystin residue, from which the tyrosin and leucin have been extracted, is now suspended in about 500 cc. boiling water and sufficient concentrated hydrochloric acid added to dissolve the cystin; the solution is decolorized with charcoal and the filtered hot solution neutralized with strong sodium hydrate. From the hot solution the cystin begins to crystallize out in large hexagonal plates as a white sandy precipitate before the neutral point is reached. It is better to filter this cystin off while the solution is acid, though a little more cystin can be obtained by exactly neutralizing. This last, however, is less pure than the first. The solution is sucked off and the crystals are washed repeatedly in cold water. They may be further purified by recrystallization. By this means one easily obtains about forty grams of crystalline, clean cystin from a kilogram of dried horn. An additional two to four grams may be obtained from the water extract containing the tyrosin and leucin by fractional crystallization, giving a total yield of about 42 grams to a kilo of dried horn. This method we have found superior to the original Mörner method of fractional precipitation from an ammoniacal solution in its ease, quickness, and the clean way in which the cystin crystallizes from a hot solution in hydrochloric acid when the latter is neutralized by sodium hydrate.

The cystein was made from the cystin thus obtained by reduc-

tion with tin and hydrochloric acid and was used throughout as the crystalline hydrochloride which had been recrystallized twice from water.

In all the following experiments sufficient cystein hydrochloride was weighed out to give 2 grams to a flask, the whole neutralized to amphoteric reaction to litmus, and the solution divided among the different flasks so that each flask contained 50 cc. of solution and 2 grams of cystein hydrochloride. The flasks were those described in a previous paper;[1] they were provided with mercury manometers, were air tight, and the oxidation was measured by the negative pressure in the flask due to the absorption of oxygen. The flasks were shaken vigorously throughout the experiment at room temperature in a shaking machine.

A. The influence of the reaction on the speed of oxidation.

TABLE I.

Influence of acidity and alkalinity on the oxidation of cystein.

Time shaken in minutes.	ABSORPTION OF OXYGEN IN MM. Hg BY CYSTEIN AT DIFFERENT ALKALINITIES.						
	Acid to congo.	Neutral to congo.	Neutral to alizarin.	Litmus amphoter blue.	Phenol-phthalein rose.	Phenol-phthalein pink.	Temp. °C.
27	0	0	3	8	10	9	21
57	0	0	9	17	16	15	21.4
120	0	0	18	37	36	28	20.4
180	0	0	26	55	49	38	22
240	0	0	31	66	60	46	20.6
277	0	0	34	73	66	49	21.8

TABLE II.

Time shaken,	ABSORPTION OF OXYGEN IN MM. Hg BY CYSTEIN AT DIFFERENT ALKALINITIES.						
	Congo neutral.	Alizarin neutral.	Litmus red-violet.	Litmus violet.	Litmus blue-violet.	Temp. °C.	Barom. mm.
15	0	0	1	1	3	23	757.5
45	0	4	5	8	12	21.7	757.5
60	0	4	6	10	13	21.4	757.5
90	0	6	10	17	21	21.2	757.5
120	0	9	15	24	27	21	757.4
165	0	13.6	23	33	38	21	756.5
220	0	18	30	40	49	20.8	756.5

[1] Mathews: this *Journal*, vi, p. 3, 1909.

TABLE III.

Time.	ABSORPTION IN MM. Hg AT CONCENTRATION OF NaOH.			
	$\frac{N}{100}$.	$\frac{N}{10,000}$.	Temp. °C.	Barometer.
33	2	5	20.5	760.7
73	6	16	21	760.7
185	11	33	22	761

TABLE IV.

*Velocity constants * of oxidation* $\frac{1}{t} \log \frac{A(B-x)}{B(A-x)} = K$

Time in minutes.	K_1.	K_2.	K_3.	K_4.
57	0.000084	0.000246	0.000230	0.000215
120	0.000124	0.000293	0.000289	0.000208
184	0.000124	0.000319	0.000262	0.000198
240	0.000122	0.000334	0.000287	0.000195
277	0.000118	0.000352	0.000289	0.000190
Mean K	0.000114	0.000309	0.000271	0.000201

K_1, neutral to alizarin; K_2, litmus blue amphoter; K_3, phenolphthalein rose; K_4, phenolphthalein pink.

* A fair constant is obtained by the use of the velocity equation of the second order. As the object was to represent numerically the relative speeds of oxidation at different alkalinity and the method without careful control of the temperature was not accurate enough to work out the mechanism of the reaction, we did not attempt to see whether an equation of the third order would, as seemed more likely, give a better constant.

The concentrations of hydrogen ions in the solutions was inferred from their reaction to different indications as shown by Salm.[1]

The results obtained and tabulated in Tables I–IV are shown graphically in the plots in Fig. I and II. It will be seen from these tables and plots that this oxidation is extraordinarily dependent upon the alkalinity of the solution. *The spontaneous oxidation of cystein takes place with any appreciable rapidity only within the limits ordinarily designated as neutrality.* It extends only from the neutral point of alizarin to just beyond the neutral point of phenolphthalein. Yet within these narrow limits there is a wide variation as may be seen in Fig. II. In this chart the

[1] Salm: *Zeitschr. f. physikal. Chem.*, lvii, pp. 471-501, 1906.

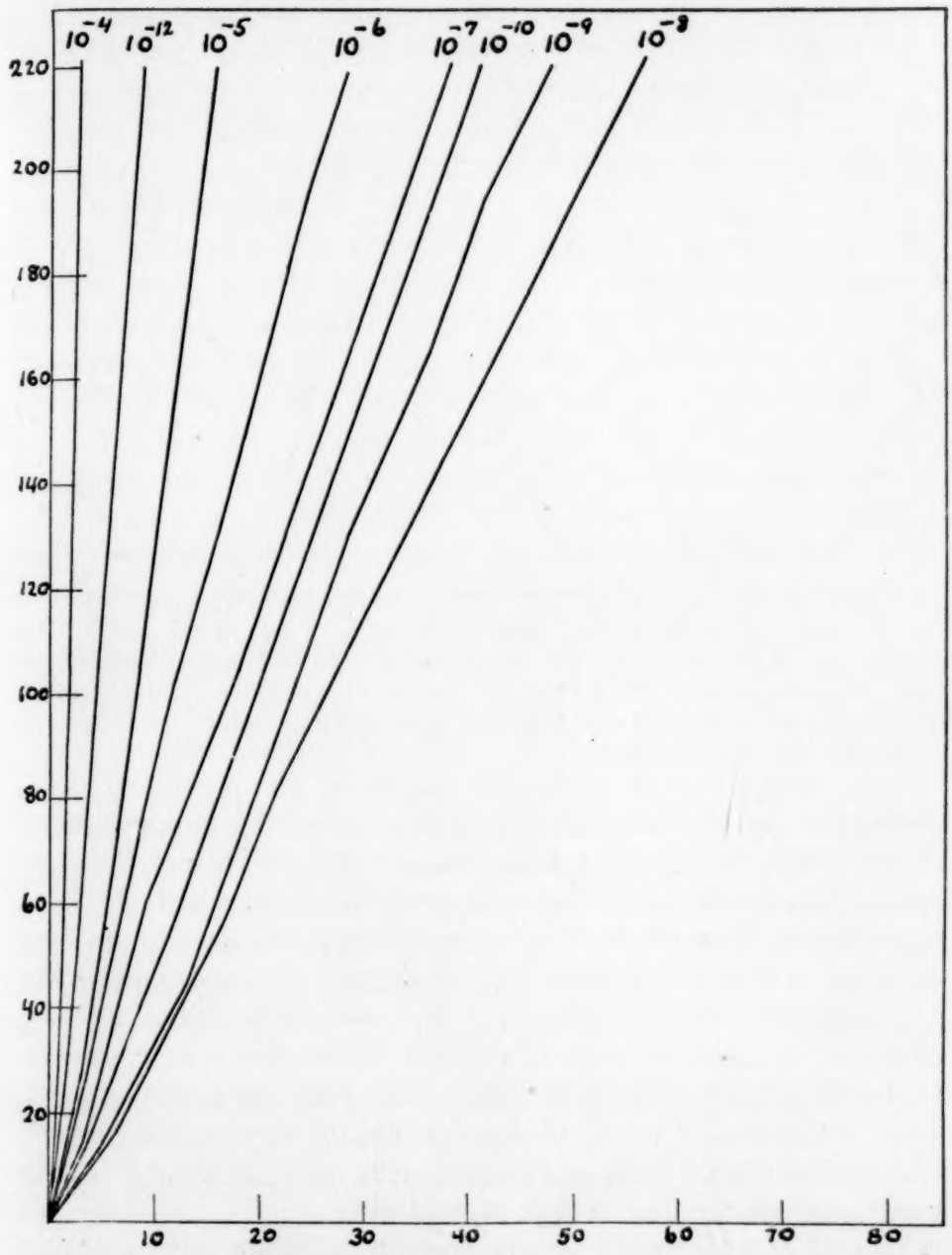

Fig. I. Rate of absorption of oxygen by cystein at different acidities. Ordinates = Time in minutes; abscissas = absorption of oxygen as represented by mm. of negative pressure of Hg. Figures at the top of the curves represent concentration of H ions × N.

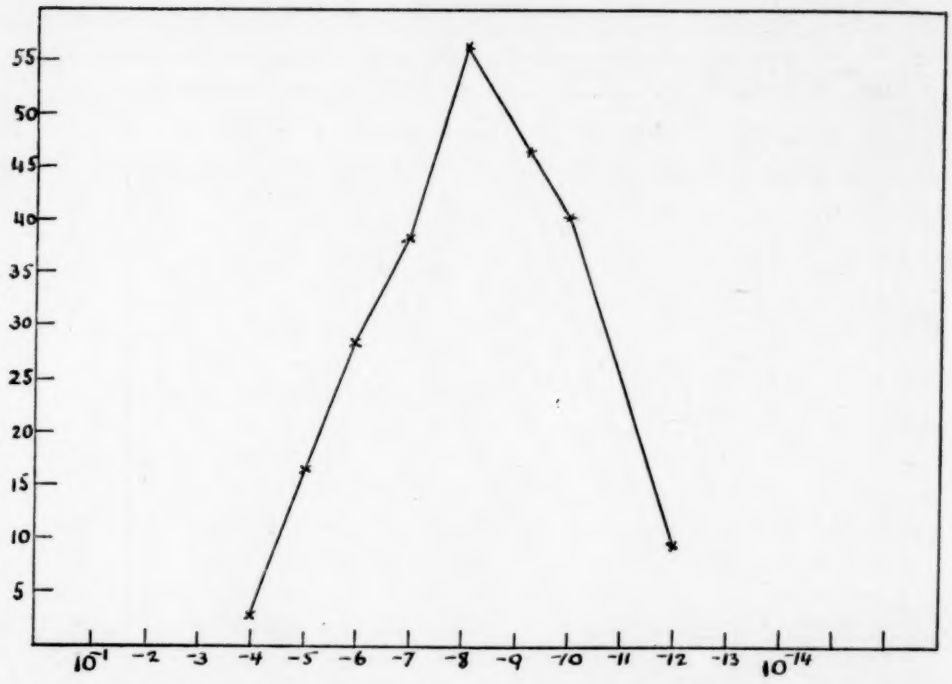

Fig II. Rate of absorption at different concentrations of hydrogen ions. Concentrations of H ions × N along the abscissa; absorption of oxygen as represented by mm. negative pressure of Hg along the ordinates after 220 minutes of shaking.

concentration of hydrogen ions is plotted on the abscissa, each division of which passing from left to right diminishes the concentration ten times. The neutral point of distilled water is approximately at 10^{-7}. The ordinates represent the millimeters of pressure due to the absorption of oxygen from the flasks after 220 minutes. The absorption is greatest under the conditions of the experiment between the limits of CH ion N 10^{-5} on one side and 10^{-10} on the other. The maximum lies at about 10^{-8}. That is a reaction which is slightly blue to litmus, but acid to phenolphthalein. This is approximately the alkalinity of the blood and presumably that of protoplasm.

In this extraordinary sensitiveness to alkalies this oxidation is, so far as we know, peculiar. It closely parallels the condition in living cells where the oxidations are also extremely sensitive to such variations and in which there is the same optimum of oxidation at this alkalinity.

The cystein oxidation shows also two other remarkable resem-

blances to living oxidations in its sensitiveness to cyanides and in its great acceleration by the least trace of iron. These peculiarities will be considered in the next two papers. These facts closely parallel the metabolic reactions of protoplasm which are also known to be closely dependent on alkalinity. We may therefore turn to the explanation of this phenomenon in cystein, with particular interest.

The great sensitiveness of the oxidation of cystein at the neutral point is explained as follows. Cystein, like all the amino-acids combines both with acids and bases to form salts. When it is the salt of an acid the cystein dissociates as a positive ion; when it is the salt of a base, it dissociates as a negative ion. It is only when it is in the neutral state that it oxidizes. It is probable, therefore, since the oxidation is almost suppressed in acid or alkaline solution, where the negative and positive cystein ions are in greatest abundance, that these ions do not oxidize or do so at an extremely slow rate. The ionization of the cystein, therefore, increases its stability and diminishes the ease of its oxidation. Since it is neither the positive nor negative ion which oxidizes, it must be something else and most probably the undissociated cystein molecule. We may assume that when the part of the molecule containing the sulphur is either electro-negative or electro-positive the electrical conditions of the sulphur do not permit it to unite with the oxygen or at any rate to lose its hydrogen. In a neutral condition we may assume either that the hydrogen comes away more easily or that the residual valencies of the sulphur atom come into activity.

SUMMARY.

(1) Cystein oxidizes itself spontaneously by atmospheric oxygen at 20–22° C. at a rapid rate, passing over into cystin.

(2) This oxidation is extremely sensitive to the reaction of the medium going only with any speed within the limits of neutrality of alizarin on one side to just beyond the alkaline side of phenolphthalein on the other. The maximum is at a concentration of hydrogen ions of about N 10^{-8}. That is between the neutral point of litmus and that of phenolphthalein. At about this point very small changes make a great difference in the speed of oxidation. This is about the reaction of blood.

(3) In this susceptibility and the position of the optimum cystein oxidation resembles protoplasmic oxidation.

(4) The most probable explanation of the susceptibility of the oxidation to the reaction is that both the negative and positive cystein ions are stable and that it is only the neutral non-ionized molecule which oxidizes rapidly, probably owing to the opening up of residual valencies of the sulphur, or the greater ease of detachment of the hydrogen under these conditions.

THE ACTION OF CYANIDES AND NITRILES ON THE SPONTANEOUS OXIDATION OF CYSTEIN.

By A. P. MATHEWS and SYDNEY WALKER.

(*From the Biochemical Laboratory of the University of Chicago.*)

(Received for publication, January 23, 1909.)

It is well known that the cyanides and the nitriles when given to mammals leave the body in part as sulfocyanides. This suggests that they combine with the labile sulfur group of the albumins of the cell, and it was this fact which led us to undertake a study of the spontaneous oxidation of cystein. It is not necessary to recapitulate the evidence that the cyanides act on cell-respiration, since all observations are practically unanimous that they do.[1] Carlson's[2] observation that the heart ganglion of Limulus may be recovered from cyanide poisoning by washing out the ganglion with hydrogen, while in itself very interesting in no way invalidates this conclusion, since the probable identity of aërobic and anaërobic respiration would lead one to anticipate his results.

The action of potassium cyanide on the spontaneous oxidation of cystein is striking. Very small amounts of the cyanide check this oxidation while larger quantities inhibit it completely for a time, although ultimately, possibly owing to the oxidation of the cyanide, the cystein escapes from the retarding action and proceeds slowly to oxidize. This is true for the spontaneous oxidation both when catalyzed by iron and when not catalyzed. Nitriles also check this oxidation.

The experiments were performed in the same manner as described in the preceding papers.[3] The readings of the manometer was made at ten or fifteen minute intervals. Two grams of cystein were in each flask in 50 cc. of solution and the reaction was always carefully noted and in general was a blue violet to

[1] See Richards and Wallace: this *Journal*, iv, p. 179, 1908.

[2] Carlson: *Amer. Journ. of Physiol.*, xix, p. 223, 1907.

[3] Mathews and Walker; this *Journal*, vi, p. 21, 1909.

TABLE I.

Manometer readings in mm. Hg. + or − refers to positive or negative pressure in flasks.

Time. *Jan. 7*	Flask I. 50 cc. H_2O + 0.1 gr. KCN.	Flask II. 50 cc. H_2O + 2 gr. cystein. No cyanide.	Flask III. 2 gr. cystein + 0.1 gr. KCN 50 cc. H_2O.	Temperature.	Barometer.
1:10	0	0	0	19.7	743.7
1:20	+ 4	− 2	+ 6	19.8	743.5
1:40	+ 8	− 9	+ 9	20	743
2:00	+ 9	−20	+10	20.5	743
2:20	+ 9	−30	+10	20.5	742.8
2:40	+ 8	−37	+ 9	20	742.8
3:00	+11	−43	+12	20.7	742.8
3:20	+10	−51	+11	20.4	742.5
3:40	+12	−54	+13	20.8	742.5
Not shaken until next day.					
Jan. 8. Began to shake at 8.42 a.m.					
8:40	+11	−62	+13	21.5	740.6
9:07	+13	−67	+11	21.2	740.5
11:05	+10	−85	+ 6	19.7	740.1
12:00	+12.9	−86.4	+10	20.5	739.2
2:55	+15	−87	+16	20.5	735.0
Not shaken until next day.					
Jan. 9. Began shaking at 11:35.					
11:35	− 2	−104	− 6	19.0	740.7
2:07	− 2	Discontinued	−18	19.0	743.0
3:12	− 3	Heavy precipitate of cystin	−24	19.1	743.3
4:15	− 5		−32	18.5	744.5
Not shaken until the next day.					
Jan. 10. Began shaking at 9:35.					
9:56	− 7		−47	19.8	750.0
10:40	− 7.5 Faint odor of KCN.		−51.5 No odor of KCN. Heavy ppt. of cystin Test for KCNS negative.	19.8	751.5

TABLE II.

Effect of small quantities of KCN. 50 cc. of solution in each flask.

Time. *Jan. 14*	Flask I. 2 grams cystein + 1 cc. $\frac{N}{2}$ KCN.	Flask II. 0.5 cc. $\frac{N}{2}$ KCN + 2 grams cystein.	Flask III. 0.1 cc. $\frac{N}{2}$ KCN + 2 grams cystein.	Flask IV. Control 5 cc. $\frac{N}{2}$ KCN.	Temperature.	Barometer.
11:00	0	0	0	0	20.0	750.6
11:22	1	+ 2	− 1	0	20.0	750.6
12:10	0.5	+ 2	− 5	+ 1	19.9	749.8
1:55	− 2	+ 3	− 8	+ 4	20.1	749.2
3:00	− 1	− 2	−15	+ 1	19.0	748.7
4:36	7	− 7	−20	− 4	18.8	748.4
	Slt. Ppt.	Ppt.	Heavy ppt.			
5:15	− 3	− 4	−20	+ 1	19.8	748.3

Not shaken during night. Began shaking next day at 8:30.

Jan. 15

8:30	− 7	− 9	−28	?	18	741.0
8:40	− 5	− 9	−28	+ 6	18.8	741.0
9:53	− 2	− 4.5	−27	+16	21.1	741.0
10:50	−12	−14	−36	+13	20.8	741.0
12:00	−19	−20	−45	+15	20.8	740.0
2:10	−40	−42	−68	+14	20.3	739.5
4:00	−60	−64	−86	+ 6	18.8	740.0

Precipitate of cystin in each flask dried and weighed.

No. 1. Wt. cystin 0.552 grams.

No. 2. Wt. cystin 0.605 grams.

No. 3. Wt. cystin 0.745. Slight loss in determination.

TABLE III.

Effect of small quantities of KCN. 50 cc. solution in each flask.

Time. *Jan. 16*	Flask I. $\frac{N}{2000}$ KCN. Containing 2 grams cystein.	Flask II. $\frac{N}{5000}$ KCN. Containing 2 grams cystein.	Flask III. $\frac{N}{10000}$ KCN. Containing 2 grams cystein.	Flask IV. $\frac{N}{2000}$ KCN. No cystein.	Temperature.	Barometer.
10:57	0	0	0	0	19.0	755.0
11:42	− 1	− 8.5	− 8.5	0	19.0	755.0
12:55	− 7	−18	−22	0	18.8	755.0
2:50	−17	−32	−40	1	19.3	755.0
3:20	−19	−35	−44	2	19.6	755.0
4:54	−28	−50	−61	1.5	18.8	754.9

TABLE IV.

Influence of $\frac{N}{100000}$ KCN.

Time. *Jan. 17*	Flask I. $\frac{N}{100000}$ KCN. Containing 2 grams cystein.	Flask II. Cystein alone.	Temperature.	Barometer.
12:10	0	0	19.8	745.1
12:58	−12	−13	19.8	745.1
2:10	−30	−30	20.4	745.8
3:17	−56	−56	20.5	746.0
4:25	−73.5	−74	21.0	746.7

TABLE V.

Influence of KCN in alkaline solution on oxidation of cystein.

Time. *Jan. 4*	Flask I. 2 grams cystein hydrochloride dissolved in 50 cc. $\frac{N}{10}$ NaOH.	Flask II. .1 gram KCN + I.	Flask III. $\frac{N}{2}$ NaOH 50 cc. + 2 grams cystein HCl.	Flask IV. .1 gram KCN + III.	Flask V. Control cystein alone.	Temperature.	Barometer.
12:07	0	0	0	0	0	20	756.9
12:55	−18	0	1	0	− 8	20.1	756.7
1:55	−35.5	0	−2	0	−20	20	755.2
2:55	−50	0	−4	0	−30	19.8	755
8:55 a.m. next day							
	−48	+1	+1	+6	−29	18.5	734.2

litmus and colorless to phenolphthalein. The experiments were made at room temperature and the flasks shaken vigorously throughout. The results of some of the experiments are given in Table I.

An inspection of the Tables I–V or the chart in Fig. I shows that potassium cyanide even in very small amounts is a strong poison for this oxidation. One-tenth of a gram of cyanide completely prevents the oxidation of 2 grams of cystein for more than two days, but at the end of that time absorption begins and then goes on slowly. Smaller quantities of cyanide reduce the rate without stopping the reaction completely. At the end of the

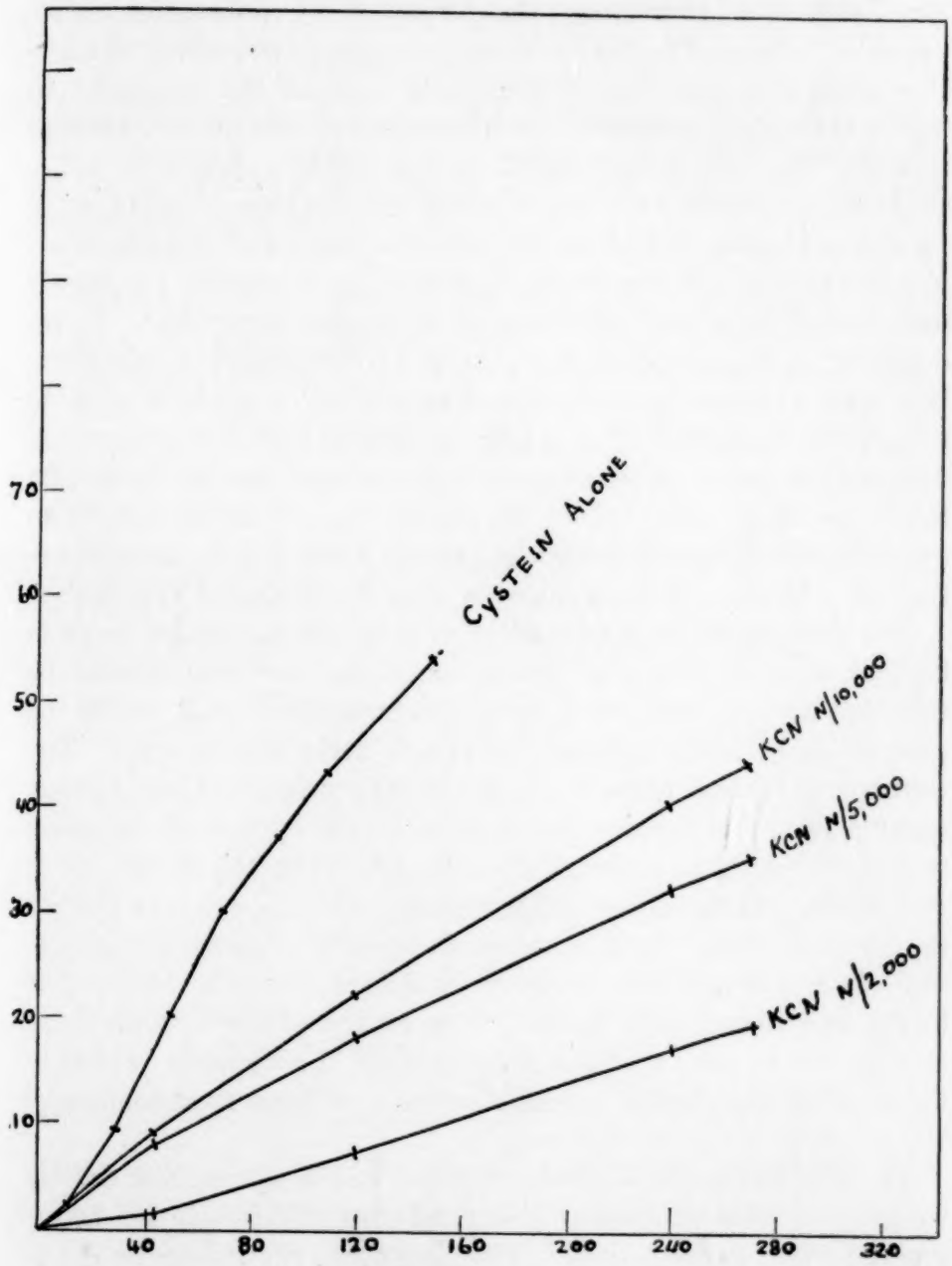

Fig. I. Curves showing the velocity of absorption of oxygen from air by cystein in the presence of varying amounts of potassium cyanide. Ordinates represent mm. of Hg negative pressure in the flasks due to absorption of oxygen; abscissas represent time in minutes.

experiment all odor of cyanide was gone, but there was no reaction with iron indicating the presence of potassium sulfocyanide. Table IV shows that in $\frac{N}{100000}$ potassium cyanide the oxidation goes on at the same rate as the control. In Table III $\frac{N}{10000}$ potassium cyanide reduces the rate of absorption for the first hour about 50 per cent; $\frac{N}{20000}$ and $\frac{N}{40000}$ potassium cyanide have no influence on the rate of absorption. In this oxidation, therefore, there is no evidence of the accelerating effects of small quantities of cyanide as observed by Kastle and Loevenhart for oxidation of hydrogen peroxide.[1] If we consider $\frac{N}{10000}$ potassium cyanide as the limit of efficiency this means that 0.35 milligram of potassium cyanide is able to retard the oxidation of 2 grams of cystein hydrochloride when dissolved in 50 cc. of solution fully 50 per cent and at the outset far more than this. Here we have, for example, potassium cyanide inhibiting the oxidation greatly when it is in the proportion of 1 of potassium cyanide to 2500 molecules of cystein.

The most probable explanation of this action appears to us to be that the proportion of cystein molecules in a condition to be oxidized at any instant of time is extremely small; while the proportion of active potassium cyanide molecules is large. The number of active oxygen atoms is also small. If we further assume that the cyanide combines with the cystein at the place where the oxygen ordinarily unites, i.e., with the sulfur atom, the results obtained are easily understood. As cyanide readily unites with sulfur compounds, as the odor of cyanide disappears and since the oxygen certainly combines with the sulfur this hypothesis is not improbable. The escape of the cystein from the inhibition and the disappearance of the cyanide probably means that the cystein cyanide compound dissociates easily and the cyanide is slowly oxidized.

The effect of the nitriles investigated was in all respects similar to that of the cyanides. We tried the effects of the lacto-mandelic and capro-nitriles. Of these three the mandelic is the most toxic to animals, the lactic the next and the capro-nitrile the least toxic.[1] This variation is owing to the fact that they decompose with varying ease and liberate hydrocyanic acid, the

[1] Kastle and Loewenhart: *Amer. Chem. Journ.*, xxix, p. 411, 1903.
[2] See Verbrugge: *Arch internat. de pharmacodynamie*, v, p. 161, 1899.

mandelic being the least stable. The results are given in Table VI.

TABLE VI.

The influence of the nitriles on the oxidation. Each flask contained 50 cc. of solution, blue-violet to litmus and containing 2 grams of cystein hydrochloride. In addition three drops of the nitriles were added. The nitriles used were obtained from Kahlbaum.

Time. *Dec. 1*	Flask I. Lacto-nitrile.	Flask II. Mandelic nitrile.	Flask III. Capro-nitrile.	Flask IV. Cystein alone.	Temper-ature.	Barometer.
1:30	0	+ 1	0	0	20.2	755.1
1:45	− 1	+ 2	+ 1	0	21	755.1
2:00	− 2	+ 3	− 2	− 1	22	755.1
2:31	− 8	+ 4	−12	− 8	22	
2:45	− 8	+ 6	−16	−10	22.5	756
3:00	− 8	+ 6	−18	−13	23	
3:25	−15	0	−30	−24	21	757.6
3:45	−14	+ 3	−35	−27	22	
4:02	−13	+ 4	−36	−28	22.2	
4:17	−12	+ 5	−39	−32	22.2	
4:30	−13	+ 4	−42	−34	22	
Not shaken during the night.						
Dec. 2						
8:30	−33	−16	−67	−56	19.5	763.5
8:56	−35	−16	−70	−60	19.5	763.5
9:30	−33	−12	−70	−63	20.0	763.5
11:10	−30	−10	−77	−69	20.5	763.5
	Fair ppt. cystin	No ppt. cystin	Heavy ppt. cystin	Heavy ppt. cystin.		

From this experiment it appeared that the capro-nitrile might have a slight accelerating action although the difference between the control and the capro-nitrile was not great and most of it came in the first hour. This led us to suspect a leak in the flask and a repetition of the experiment showed the capro-nitrile to be inert and not to stimulate.

The experiment recorded in Table VII shows that the capro-nitrile has no effect on the oxidation of cystein; that the lacto-

TABLE VII.

Conditions the same as in the preceding experiment.

Time. *Dec. 10*	Flask I. Capro-nitrile.	Flask II. Lacto-nitrile.	Flask III. Mandelic nitrile.	Flask IV. Cystein alone.	Tempera-ture.	Barometer.
3:25	0	0	0	0	20.5	745
3:45	− 4	− 2	0	− 2	20	745
4:00	−10	− 4	0	− 8	19.8	
4:40	−20	− 3	0	−18	20	
5:00	−22	− 3	0	−20	20	
Not shaken during night.						
Dec. 11						
8:20	−36	− 7	− 6	−39	18.9	743
Dec. 14.						
9:00	−77	−34	−28	−76	20.4	741.0
Dec. 16.						
12:00	−114	−72	−54	−112	19	751

nitrile retards it and the mandelic nitrile retards still more. It is probable that the amount of hydrocyanic acid formed by the dissociation of the capro-nitrile is so small as not to hinder the oxidation.

The well known toxic action of cyanides toward the catalysis of hydrogen peroxide by iron aroused the suspicion that there was always a trace of iron in our preparations and that the cyanide was acting on that. We have purified the cystein with the greatest care by repeated recrystallization from water and hydrochloric acid so that it gives not the faintest trace of iron by the development of a violet tinge on neutralization and shaking with air. As the cyanides and nitriles acted the same on this preparation of cystein as on the others we concluded that the action is on the cystein itself.

SUMMARY.

(1) Very small amounts of potassium cyanide are sufficient to check or prevent the spontaneous oxidation of cystein to cystin both in neutral and alkaline solution.

(2) Mandelic nitrile checks this oxidation; lacto-nitrile strongly reduces its velocity, but is less efficient than the mandelic nitrile; capro-nitrile leaves the oxidation unaffected.

(3) These toxins probably act by uniting with the sulphur group of the cystein in the position that the oxygen ordinarily unites and thus check the oxidation. Possibly the cyanides unite in the cell also with the labile sulphur atom of the albumins, since they escape from the mammalian organism as sulfocyanides. Whether they check respiration solely by uniting with this sulfur or in some other way cannot be said. In any case the resemblance between the spontaneous oxidation of cystein and the respiration of the cell in their relation to alkalinity, iron, arsenic, mercury, nitriles and cyanides, is suggestive.

SOIL FATIGUE CAUSED BY ORGANIC COMPOUNDS.[1]

(Plate I.)

By OSWALD SCHREINER and M. X. SULLIVAN.

(From the Laboratory of the Bureau of Soils, U. S. Dept. of Agriculture, Washington, D. C.)

(Received for publication, January 14, 1908.)

Liebig,[2] in Letter XII of his "Familiar Letters on Chemistry," says:

A field in which we cultivate the same plant for several successive years becomes barren for that plant in a period varying with the nature of the soil; in one field it will be three, in another seven, in a third, twenty, in a fourth, a hundred years. One field bears wheat and no peas; another beans and turnips, but no tobacco; a third gives a plentiful crop of turnips, but will not bear clover. What is the reason that a field loses its fertility for one plant, the same which at first flourished there? What is the reason one kind of plant succeeds in a field where another fails?

Liebig answered these questions by saying:

Wheat, clover, turnips, for example, each requires certain elements from the soil; they will not flourish where the appropriate elements are absent. Science teaches us what elements are essential to every species of plant by an analysis of their ashes. If therefore a soil is found wanting in any of these elements, we discover at once the cause of its barrenness and its removal may now be readily accomplished.

But has science removed the causes of the barrenness of a soil by the analysis of the ashes of plants? In this connection it might be well to quote a statement from an article by Coleman, which was awarded the prize of the Royal Agricultural Society of England in 1855.[3]

The theories of scientific men led us to expect that fertility depended upon the presence of certain mineral substances which were found invari-

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[1] Presented at the Chicago Meeting of the Amer. Soc. of Biol. Chem. by permission of the Secretary of Agriculture. *Proc. Amer. Soc. Biol. Chem.*, i, no. 3, p. 120.

[2] *Liebig's Complete Works on Chemistry. Familiar Letters on Chemistry*, Letter XII, p. 35, 1852.

[3] *Roy. Agr. Soc. Journ.*, xvi, 169, 1855.

ably present in the ash of plants, and the analysis of a soil, it was believed, would confirm the practical experience of the farmer; their hopes have been falsified except in the few cases of almost simple soils, such as pure clay and sands. In all other instances the analysis presented the existence in varying proportions of those substances supposed to induce fertility equally in the barren as the fertile soil. The proportion of the various ingredients was next proposed as a sign of quality, but researches into the amount of inorganic matter abstracted by each crop have demonstrated that soils of a mixed character contain abundant supplies of mineral foods for numerous crops.

This was over fifty years ago and the statements made are practically as true today as they were then. Though analyses, not only of plants, but of soil also have been made for many years, the causes of the decrease of yield of crops grown successively have never been fully understood, nor indeed has any general remedy been found for the decrease. Thus it has been found from a consideration of a vast number of ash analyses that no certain relation exists between the ash and the plant and the fertilizer requirements of soil.[1] Hargreaves,[2] who has reviewed investigations relating to chemical methods of determining available plant food in soils states the history of such work to be a "record of comparative failure."

In many cases barrenness of soil towards one or more plants persists despite the addition of fertilizers, as Liebig well recognized, for he says:[3]

But it has been observed that the crops are not always abundant in proportion to the quantity of manure employed, even though it may have been of the most powerful kind; that the produce of many plants, for example, diminishes in spite of the apparent replacement by manures of the substances removed from the soil, when they are cultivated on the same field for several years in succession.

From the above quotation it may be seen that Liebig recognized that there are many cases which his theory of mineral requirements failed to cover. Indeed, if he had followed the idea embodied in the quotation to its logical analysis, he would have come to the conclusion brought forth in the present paper

[1] See in this connection Tollens: *Expt. Sta. Record*, xiii, pp. 206 and 305, 1901.

[2] *Journ. Dept. Agr. So. Australia*, x, p. 420, 1907.

[3] *Liebig's Complete Works on Chemistry*, part i, p. 55, 1852.

as regards one of the causes of the decrease in yield of succeeding crops.

At the present day it is a matter of common knowledge in agriculture that when one crop is successively grown upon the soil, the yield usually shows a decline which even the use of fertilizers does not completely offset. In the course of time, indeed, the land may become unfit for the growth of that plant, but another plant will thrive where the former failed. When a soil is in such a condition that it will not give a good yield or a normal yield of certain crops, it may be said to be fatigued or sick. Thus we have clover-sick soils, cowpea-sick soils, flax-sick soils, etc.

Though there might be soils so run down that they will not give for any crop a normal yield, or a yield approximately what they formerly gave, in this paper we shall use the phrase "sick soil" to denote that the soil in question is in such a condition that it gives a much poorer yield of a certain kind of plant than it formerly did, while still giving a normal yield of some other crop. The cause of the soil-sickness has been attributed to the following fundamental factors: (1) the poverty of the soil in certain plant foods or to improper relations of nutritive elements brought about by selective plant absorption; (2) injurious organisms, such as bacteria, molds, worms, etc.; (3) poor mechanical condition of the soil as a result of the one crop system; to these must be added, (4) special toxic conditions due to the accumulation of toxic organic compounds. This accumulation may be either the result of direct excretion from the plant or of secondary changes of plant excreta and plant débris, through the oxidation or reducing action of soils, or as a result of the growth of microörganisms, especially bacteria and molds.

In regard to direct excretion it should be said that the theory that plants excrete from their roots substances which are deleterious to continued growth, was promulgated by DeCandolle[1] as early as 1832.

DeCandolle formed a theory to explain the well-known fact that continuous cropping of the same plant often results in decreased growth, while a good growth of every crop may be obtained if rotation is resorted to. He distinguishes between

[1] *Physiologie végétale*, Paris, 1832.

true exhaustion of the soil, in which case a soil is to be considered as depleted in respect to the soluble salts necessary for plant growth, and what may be termed false exhaustion, in which case the cause of the poor crop is to be looked for in the presence of injurious excretions from former crops. DeCandolle's theory of crop rotation is then that crops, through their root excretions, render the soil unsuitable for the same, or closely related plants, but that these root excretions are harmless, or even beneficial to distantly related plants.

The ideas and observations of DeCandolle were not allowed to pass unchallenged, for within a few years controversial articles began to appear.

DeCandolle's theory rested upon an inadequate foundation of actually observed facts with little experimental demonstration. Indeed, in the light of modern methods of experimentation, the work of Macaire-Princep[1] and Brugman,[2] upon which it rested to a great extent, is not to be considered real evidence. The later works of Unger,[3] Braconnot,[4] Walser,[5] Boussingault,[6] Gyde,[7] and others are likewise mainly of historical interest because the insufficiency of their methods and the difficulty of the task renders their results such that they can not be accepted as evidence for or against excretions or the toxic or non-toxic nature of these.

Liebig, however, at one time supported DeCandolle's theory of crop rotation, as is shown by the following statement, quoted from Gilbert:[8]

> The first definite theory as to the benefits of the alternation of crops assumed that the excreted matter of one description of crop were injurious to plants of the same description, but they were not so, and might even be beneficial to other kinds of plants. At first Liebig pronounced this theory of rotation to be the only one having any really scientific basis.

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[1] Memoire pour servir à l'histoire des assolemens, *Mém. de la soc. de physique et l'histoire nat. de Génève*, v, p. 282, 1832.

[2] *De mutata humorum in regno organico indole*, 1786.

[3] *Einfluss des Bodens auf die Vertheilung der Gewächse*, Vienna, 1836.

[4] *Ann. chim. phys.*, lxxii, p. 27, 1839.

[5] *Unters. ü. d. Wurzelausscheidungen*, Tübingen, 1838.

[6] *Rural Economy*, trans. by Law, p. 345, London, 1845.

[7] *Trans. Highland Agr. Soc.*, p. 273, 1845–7.

[8] Agricultural investigations at Rothamsted, England, during a period of fifty years, Bull. 22, Office Exp. Stat., U. S. Dept. Agr., p. 174, 1805.

Later he seems to have modified his view considerably; and to have supposed that the explanation was, not that the excreted matters of one description of plant were injurious to another of the same description, but that, as the different plants had such very different mineral requirements, the alternation of one kind with another relieved the soil from exhaustion.[1]

Later, however, Liebig brought forth the idea that a successful growth of plants requires a certain amount and proper ratio of easily soluble mineral nutrients in the soil; that plants could no more attain their maximum growth in the absence of a proper ratio of these mineral nutrients than when the total quantity was too small. This theory has become known as Liebig's Theory of Mineral Requirements. On this basis crop rotation is explained as follows: Since plants have different mineral requirements, continued cropping with any given kind of plant destroys the necessary ratio; but an alternation of different kinds of plants relieves the soil of exhaustion.

Aided by the weight of his great authority Liebig fixed his theory upon the masses, and it became the established law in the practice of agriculture. With the establishment of Liebig's theory of mineral requirements, the theory of toxic excretions was lost sight of, or considered absurd until it was shown that the presence of toxic bodies in soils, be it the result of plant excretion or otherwise, has considerable foundation in fact.

The isolation of several organic bodies from soils has already been reported upon.[2] One of these bodies, identified as picoline carboxylic acid, was isolated from an unproductive soil and found to be moderately harmful to wheat, while closely related compounds, such as uvitonic acid and pyridine derivatives. were very toxic. Another of these bodies isolated from a poor soil and identified as dihydroxystearic acid was very toxic to plants. Two other bodies, agrosterol and agroceric acid, also isolated from unproductive soils, were, however, nontoxic to wheat although a fuller study of their relationship to other compounds may bring about an understanding of their origin and function in the soil. Considerable indirect evidence of the presence of

[1] *Liebig's Complete Works on Chemistry*, p. 57–8, 1852.

[2] *Journ. Amer. Chem. Soc.*, xxx, pp. 1295 and 1599, 1908; xxxi, p. 116 1909. *Science*, xxviii, p. 190, 1908.

harmful bodies in many unproductive soils has also been given in previous publications.[1] Among the indirect evidences of the presence of toxic bodies in soils the work of Pouget and Chouchak[2] on the alfalfa soils in France should also be mentioned especially in connection with the subject of the present paper.

Living things, such as bacteria, yeasts, molds, animals, and man have their activities decreased in force and even destroyed when the products of these life activities remain unchanged in their immediate environments and are again absorbed. Thus, microörganisms, growing in a solution, are changed in growth and die out, unless the products of their metabolism are altered, destroyed, or taken away.

Eijkman[3] has found that bacteria and molds, growing on nutrient gelatine or nutrient agar, form waste products which inhibit growth. These inhibiting substances are diffusible, but could not be filtered through a porcelain filter and are destroyed at the temperature at which the organisms are killed. The waste products of a given species he found to be more toxic, as a rule, to that and closely related species, than to those more distantly related.

According to Rahn[4] bacteria first multiply rapidly in bouillon, later the increase in number is slower, and finally the bacteria cease to multiply. Old cultures, sterilized by heat or chloroform gave good growth on reinoculation, but when sterilized by ether the old culture broth gave no growth when reinoculated. Fresh bouillon shaken with ether and inoculated gave good growth. In the case of *B. fluorescens* Rahn found that the inhibition was due to thermolabile substances which could not be filtered through a porcelain filter and were not destroyed by ether.

More recently, Kuester[5] found that molds growing in a solution produced substances which inhibited the growth of a second inoculation. The inhibitory substances were destroyed by boiling.

In the case of man and animals it is well known, to give but a

[1] Bulletins 28, 36 and 40, Bureau of Soils, U. S. Dept. Agric.
[2] *Compt. rend., d. l'acad. d. sci.*, Paris, cxlv, p. 1200, 1907.
[3] *Centralbl. f. Bakt.* I, xxxvii, p. 436, 1904.
[4] *Centralbl. f. Bakt.* II, xvi, p. 417, 1906.
[5] *Ber. d. d. Bot. Gesell.*, xxvi a, p. 246, 1908.

few examples, that accumulation of waste, such as urea, uric acid, etc., in the system leads to speedy death, and that the rebreathing of exhalations from the lungs leads to distress. Bodily fatigue, likewise, is due not to exhaustion of food in muscles, blood and lymph, but rather to an excess of products of metabolism. Thus Mosso[1] found that blood taken from a tired dog would, on injection into a fresh dog, bring about in the latter all the signs of fatigue. Weichart[2] isolated from fatigued muscles a true toxin of a chemical and physical nature like bacterial toxins. When injected into animals this toxin gave rise to the phenomena of fatigue. Lee[3] found that lactic acid occurring normally in muscles and blood, and its sodium and potassium salts, and oxybutyric acid occurring in diabetes mellitus would cause fatigue when injected into the muscles or blood of animals. More recently Slade[4] found that extracts from muscles, especially from fatigued muscles, injected into animals would produce fatigue and exhaustion.

For the purpose of studying the cause of soil fatigue when plants are grown successively on the same soil, wheat and cowpea, as types of the gramineous and leguminous plants respectively, were grown on different soils until the yield became poor. The present paper deals with the study of one of these soils to determine the cause of the unproductivity.

Cowpeas were grown in the greenhouse upon a sandy loam soil. At first the cowpeas grew finely upon this soil, but as the number of recroppings increased the soil came to such a condition that it would no longer give even a fair crop of this plant. Since wheat and potato grew well on this soil after the failure of cowpea, it seemed that the poor growth could not be due to lack of plant nutrients. Furthermore, the water extracts of this soil contained 890 parts per million of total solids and 500 parts of soluble nitrates, reckoned on the weight of the moist soil. The water extracts were slightly alkaline after boiling to drive off the carbon dioxide. It would seem then that the decreased yield of cowpea was due neither to lack of plant nutri-

[1] *Arch. f. Anat. u. Physiol.*, 1890, Physiol-Abt., p. 89.
[2] *Münch. med. Wochenschr.*, li, p. 2121, 1904.
[3] *Journ. Amer. Med. Assoc.*, xlvi, p. 1491, 1906.
[4] *Journ. of Physiol.*, xxxv, p. 163, 1907.

ents nor to the presence of acids, but rather to the presence of some toxic substance or substances. Accordingly, means were taken to discover this substance or these substances.

Before considering these means, however, it might be well to describe the methods employed in the case of cowpea culture in water and pot. Unlike wheat, cowpea germinates poorly on water, so it was found best to germinate the seed, either in sand or in finely divided blotting paper until the primary leaves are well formed and there is a good tap root. The blotting paper method gives the best tap root, but has the disadvantage that the paper adheres to the hairs of the roots. In most of the experiments with cowpea in water culture, therefore, the germination in sand was used. By moistening the sand the young cowpeas can easily be removed without injury. They are then placed in wide-mouth culture bottles with a capacity of 250 cc., being held in place by inserting their stem in V-shaped vertical grooves cut in the margin of flat cork stoppers. Each seedling is held in position by replacing the cork wedge, which had been removed in cutting the groove, sufficient cork being removed from the apical edge to give space for the stem. The cork as a whole is bound with a rubber band. For the most part a small quantity of cotton, though not absolutely necessary, was wrapped about the stem to insure greater protection to it and to aid in holding the seedling in position. Care was taken not to wet the cotton, for if the cotton becomes saturated with moisture, stem-rot of the plant may ensue.

In the water culture experiments four seedlings were used to a cork. For pot culture the seedlings were germinated between moist filter paper or, better, the cowpeas may be germinated on moist sand. In the latter case it has been found advantageous to place the seed on the surface of the sand with the hilum up. As soon as germinated the seedlings were potted, three to a paraffin wire pot[1] holding 450 grams of soil.

These pots are made from galvanized wire net, having a one-eighth inch mesh. The net is cut into strips 3½ inches by 10 inches. The ends are brought together and fastened by short rivets. At

[1] For a description of paraffin pot see Cir. 18 and Bulletins 36, 40 and 47, Bureau of Soils, U. S. Dept. Agr.

intervals along one end of the cylinder thus formed, vertical incisions, one-half inch long, are made and the ends are turned in to hold the bottom, which consists of a disk of the same material. The inverted pot is then dipped into hot paraffin to the depth of about one inch, removed to cool and redipped until a rim of paraffin is formed at the top of the pot. The wire pot is three-fourths filled with the moist soil and the loose soil particles are brushed off the outside. Then the filled pot is immersed into a bath of melted paraffin to the upper rim of paraffin until bubbles of air begin to come from the soil, when it is withdrawn, cooled, either in the air or by dipping into water, and then redipped; this operation being repeated until a substantial coating is formed. In this way the outer layer of soil is coated with paraffin; so in reality the soil is inclosed in a paraffin pot reinforced by wire.

This form of pot has given great satisfaction since the roots grow freely throughout the soil and not along the side and on the bottom, as in the ordinary pot. The seeds are planted just as soon as they are germinated, generally six to a pot in case of wheat and four in case of cowpeas and the surface of the soil is covered with a light sand to protect the soil from wash, and water is added from time to time to keep the soil at the optimum water content.

Since the amount of cowpea-sick soil was not very great, only a limited number of experiments could be made to determine whether or not the poor growth of the cowpea on the soil was due to the presence of toxic substances. It may be said, however, that these experiments, though limited, seemed quite decisive and are therefore reported in the present paper.

Since wheat and potato grew well on this soil, it seemed perfectly obvious that the failure of the cowpea was due to something in the soil of an inhibitory nature. Examinations of the soil showed that it was free from nematodes or other worms. As regards the soil as a poor medium for cowpeas, the questions naturally arose, (1) could material toxic to cowpea be extracted from the soil? (2) would the soil from which the material was extracted become thereby a good medium for cowpeas? To answer these questions the following experiments were made:

First, the soil was shaken with a large volume of water and the

water separated from the soil by means of a Pasteur-Chamberland filter. This filtrate or water extract of the cowpea soil was used as a medium in which to grow cowpeas to determine whether or not any toxic material was extracted by water. Cowpeas growing in distilled water were used as control. Normally this water extract of the soil, because of its content of plant nutrients, should give a much better growth of cowpeas than the carbon-treated distilled water. On the whole there was but little difference between the growth in the soil extract and the distilled water and while this result is indicative, the evidence from these experiments is rather inconclusive.

Passing over the further study of the soil and soil filtrate to the isolation of the body causing the soil fatigue it may be said that many of the methods for such work as devised in these laboratories were tried, and distillation with steam, perhaps the simplest and most straight-forward of these methods, gave positive proof of the presence of a crystalline body which proved to be toxic to cowpea. The method which gave this toxic body is as follows: The soil was treated with a large amount of water and the mixture placed in a large container connected with a glass condenser and a steam boiler in the manner customary for conducting steam distillations. The steam was made to pass through the soil and water, and the flask in which the soil was contained was kept hot by means of a paraffin bath to prevent the undue accumulation of water by the condensation of the steam. After the soil had been distilled with steam in this way for several hours the operation was stopped and the soil and distillate were kept.

The distillate was slightly turbid. Part of the turbid distillate was filtered and the filtrate used as a medium in which to grow cowpeas. The filtered distillate was found to retard the growth of cowpea as compared with pure distilled water. The roots of the cowpeas growing in the distillate from the soil were poorly developed and the leaves were somewhat curled and considerably smaller than the leaves of the control plants growing for the same length of time under the same conditions.

TABLE I.

Growth of cowpea in distillate from cowpea-sick soil.

	Relative green weight.
Control, distilled water	100
Distillate	66

The distillate was turbid as above mentioned, and, on standing, fine needle-shaped crystals separated from it and more crystals were obtained from the solution by extraction with ether. These crystals were dried by suction and washed with water several times. They were soluble in ether, somewhat soluble in alcohol, slightly soluble in water. They charred on heating and contained no nitrogen. Heating in a capillary tube, in a paraffin bath, they began to liquefy about 60° C. The general crystalline form is shown in the accompanying microphotograph, Fig. 1, and is very characteristic of the substance.

Some of these crystals were placed in water which was brought to boiling. The substance melted and floated on the surface of the water and clung to the sides of the beaker like tarry matter. When the water was cool it was filtered and the saturated solution of the crystals thus obtained was used as a medium in which to grow cowpeas, with distilled water as a control as outlined in the previous pages. The effect of the substance on the development of the cowpea was very marked indeed, the plant being greatly stunted both in root and top. The comparative growth is shown in the following table and in Fig. 2.

TABLE II.

Comparative growth of cowpeas in distilled water and in solutions of crystals obtained from cowpea-sick soil, by steam distillation.

		Relative green weight.
1.	Distilled water	100
2.	Saturated solution of crystals	38

The soil from which the crystalline matter was obtained was brought to its optimum moisture content by allowing the excess of water to evaporate at room temperature. This soil—which we may call the steam distilled soil—and the original cowpea-sick soil were placed in paraffin pots and seeded with newly germinated cowpeas and the pots were placed side by side in the

greenhouse. Without going further into details, it may be said that the soil from which the distillate was made was vastly better as a medium in which to grow cowpea than the untreated soil. The improvement in the soil is clearly seen from the following table:

TABLE III

Comparative growth of cowpea on untreated and steam distilled cowpea-sick soil.

		Relative green weight.
1.	Soil, untreated	27 or 100
2.	Soil, steam distilled	100 or 369

Since material toxic to cowpea was extracted from the cowpea-sick soil and since the soil was thereby made a good medium in which to grow cowpeas, it must be concluded that the decreased yield of the cowpea on the soil in question was due to toxic matter in the soil. Whether this toxic matter was deposited therein by the plant and accumulated with the successive croppings, or was due to toxic matter arising in the decay of whatever plant débris might be left in the soil by each crop is left undecided by these experiments.

The experiments do show that organic substances arising through crop growth, whatever may be their specific origin, can cause soil fatigue or infertility as illustrated by the present case, although the other factors mentioned in this paper may obtain in other soils or under other conditions.

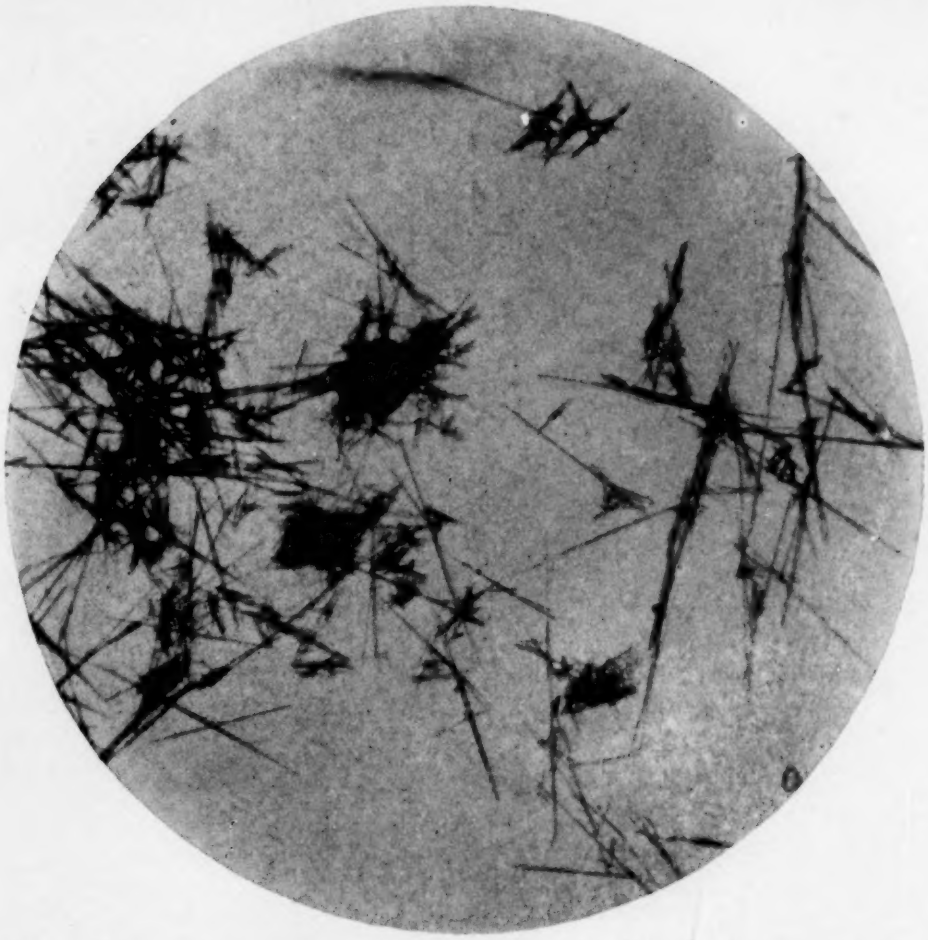

FIG. 1 MICROPHOTOGRAPH OF CRYSTALLINE SUBSTANCE EXTRACTED FROM A SOIL FATIGUED BY CONTINUOUS CROPPING WITH COWPEAS.

FIG. 2. EFFECT ON COWPEAS OF THE CRYSTALLINE SUBSTANCE EXTRACTED FROM SOIL FATIGUED BY CONTINUOUS CROPPING WITH COWPEAS: (1) CONTROL IN PURE DISTILLED WATER; (2) SOLUTION OF THE CRYSTALLINE SUBSTANCE IN PURE DISTILLED WATER.

A NOTE ON THE PREPARATION OF GLYOXYLIC ACID AS A REAGENT.

By STANLEY R. BENEDICT.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*)

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Hopkins and Cole[1] have proposed the employment of a solution of glyoxylic acid as a substitute for the glacial acetic acid used in the Adamkiewicz reaction for the tryptophane grouping in protein or other substances. In the preparation of their reagent, Hopkins and Cole advise the reduction of oxalic acid to glyoxylic, by means of sodium amalgam. Inasmuch as sodium amalgam is not always readily available, it may be of interest to call attention to the fact that metallic magnesium may be employed as the reducing agent for obtaining glyoxylic acid from oxalic acid. The following is the procedure. Ten grams of powdered magnesium[2] are placed in a large beaker, or preferably a large Erlenmeyer flask, and shaken up with enough distilled water to liberally cover the magnesium. Two hundred and fifty cubic centimeters of a cold, saturated solution of oxalic acid are now added slowly. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should best be cooled under the tap during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid[3] and made up to one liter with distilled water. The solution gives no reaction for oxalate with

[1] Hopkins and Cole: *Proc. Roy. Soc.* lxviii, p. 21, 1901; also *Journal of Physiology*, xxvii, p. 418, 1902, footnote.

[2] Magnesium ribbon may also be employed, but in this case the reaction goes more slowly and may be accelerated by warming.

[3] The reason for the addition of the acetic acid is that the filtrate may sometimes be faintly alkaline in reaction, and in such cases the magnesium would be partially precipitated upon long standing, unless the acid is added.

calcium and contains apparently only the magnesium[1] salt of glyoxylic acid. This reagent gives very beautifully the characteristic reactions for glyoxylic acid.

[1] The magnesium could readily be removed, but there appears to be no objection to its presence.

ON CRITICAL HYDROXYLION CONCENTRATIONS IN DIASTATIC HYDROLYSIS.

By CLARENCE QUINAN, M.D.

(From the Hearst Laboratory of Pathology, University of California.)

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Under certain conditions, some of which are fairly well worked out, diastase converts starch into sugar. In modern phraseology the phenomenon may be described as an atomic rearrangement in the starch molecule, accelerated by the presence of a specific ferment. Unfortunately, since the molecular configuration of starch is uncertain, it is only possible to indulge in conjecture as to the steps of this cleavage, though the end product, maltose, belongs in a group of bodies possessing definite structural characters. For experimental purposes it is customary to utilize diastase obtained either from saliva or pancreatic-juice, though many have preferred to work with the taka ferment. From whatever source obtained, however, it appears to possess essentially the same peculiarities, requiring for the full development of its activity an environment of favorable concentration, temperature and reaction. According to the prevailing belief, the hydrolytic cleavage of starch usually occurs in a medium slightly alkaline, and is not to be expected in the intestine, for example, until the free acid of the stomach has been neutralized. It will presently become evident that such a belief is not supported by sound experimental data.

Recently, while engaged in determining the amino-acid output in the autolysis of swine pancreas, I ventured to add starch paste to a separate portion of a digestion mass consisting of the pulped fresh gland in 0.3 per cent solution of sodium carbonate. To my surprise, after 20 hours at a temperature of 36° C. no sugar had formed, and several days later, when amino-acids were readily separated, but traces of a reducing substance could be

detected. Led by this observation, the problem was attacked by a process of elimination, and evidence was gradually accumulated pointing clearly to the fact that certain electrolytes are specifically obnoxious to diastase.

Turning now to the literature of the subject, nothing helpful was found in text-books dealing with the general problems of physiological chemistry; indeed, in most of them, no mention is made of any such peculiarity of the ferment. Taylor[1] who has carefully analyzed the literature from the point of view of physical chemistry, does not emphasize it, and the bibliography of the subject assembled by him contains little which bears directly upon this phase of the question. In Oppenheimer's monograph,[2] a reference was found from which, as a starting point I have segregated the special literature to the present time.

Speaking broadly, we may divide the twenty or more contributions which have a more or less direct bearing upon the subject, into two classes, depending upon the technique employed. In one, much the larger, sweeping conclusions are drawn from simple uncontrolled test tube experiments; those, especially, who first encountered the phenomenon, having been well content, it appears, to glean at random in fresh fields, without particular regard to the means employed. As might be expected, the data are contradictory and susceptible of almost any interpretation. In the other, smaller class, electro-chemical methods were invoked, and by this means it first became possible to bring into harmony many apparently conflicting observations, and supply a certain quality of continuity.

Grützner[3] was first, it seems, to point out that sodium carbonate, even in very dilute solutions, inhibits the action of diastase; though Nasse[4] working with 4 per cent solutions of various neutral salts, had already noted what he thought to be individual preferences on the part of different ferments for particular salts.

[1] Taylor: On Fermentation, *Univ. of Calif. Publ. Pathol.*, i, 1907.

[2] Oppenheimer: *Ferments and their Actions*, London, p. 179, 1901.

[3] Grützner: Notizen über einige ungeformte Fermente des Säugethierorganismus, *Arch. f. d. ges. Physiol.*, xii, p. 285, 1876.

[4] Nasse: Untersuchungen über die ungeformten Fermente, *Ibid.*, xi, p. 186, 1875.

After the lapse of many years, Kubel[1] a pupil of Grützner's, undertook, at his suggestion, a study of the action of various salts upon ptyalin. He was able to confirm and amplify considerably the findings of his preceptor, stating that diastase is sensibly depressed by the presence $\frac{N}{5000}$ potassium hydroxide. Struck by the importance of this he writes: "Es muss also in Zukunft der Satz gelten: Das Speichel-Ferment wird durch keinerlei alkalische Reaktion unterstützt, sondern geschädigt," a prophecy, it may be remarked, which has not been widely heeded.

Chittenden and Ely[2] were aware of the restraining action of alkali when they attempted to determine the action of peptone on starch cleavage accelerated by ptyalin. They concluded that 1 per cent of peptone, even in the presence of 0.3 per cent sodium carbonate, "causes a greatly increased formation of sugar." "It seems probable," they assert, "that in the proteid and diastatic pancreatic digestion, the formation of peptone, which goes on side by side with sugar formation, must help the process." It must be observed, however, that in spite of the thoroughness with which their work was carried out, the actual increase noted was not large enough to be impressive, though somewhat beyond the limits of error in observation.

Of recent years much attention has been paid to the effects produced by various salts and acids in promoting or impeding the action of diastase. Thus, Patton and Stiles[3] take issue with Grützner on the point that saturated solutions of magnesium sulphate and ammonium chloride depress the ferment, their results pointing quite the other way. They found that calcium salts have a restraining influence only when in concentrated solution. On the other hand, Pozerski[4] maintains that small quantities of calcium added to pancreatic juice—he added 0.3 cc. of $\frac{N}{2}$ calcium chloride to 2 cc. of juice—nullify its diastatic power.

[1] Kubel: Ueber die Einwirkung verschiedener chemischer Stoffe auf die Thätigkeit des Mundspeichels, *Ibid.*, lxxvi, p. 276, 1899.

[2] Chittenden and Ely: Influence of Peptone and Certain Inorganic Salts on the Diastatic Action of the Saliva, *Journ. of Physiol.*, iii, p. 327, 1882.

[3] Patten and Stiles: On the Influence of Neutral Salts upon the Rate of Salivary Digestion, *Amer. Journ. of Physiol.*, xvii, p. 26, 1906.

[4] Pozerski: Sur la disparition de l'amylase dans les sucs pancréatique activés par les sels de calcium, *Compt. rend. soc. biol.*, lx, p. 1068, 1906.

Delezenne[1] found that these salts have a specific accelerating influence upon the progress of tryptic proteolysis. He declares, however, that the greater part of the calcium added to the juice is utilized in the precipitation of alkaline carbonates and phosphates, and that the quantity of calcium which enters definiteliy into the phenomenon of activation is extremely small. Pozerski calls attention to the remarkable parallelism between this observation and his own.

Fernbach and Wolff,[2] studying the liquefaction of starch, reached conclusions of interest. According to them: "Les sels neutres au methyl orange n'ont aucune influence sur la perte de viscosité des empois chauffés sous pression; par contre, les sels alcalins à ce reactif gênent beaucoup la liquéfaction, et il suffit de traces d'alcalis libres pour l'empêcher. L'interêt de ces observations s'accroit du fait que la liquéfaction diastasique des empois obéit à des influences analogues." The same authors, working with a maceration of malt—4 cc. of 10 per cent malt solution to 50 cc. of 5 per cent starch—found, that calcium salts were without action even when present in considerable quantity.

Bierry and Giaja[3] discovered that pancreatic juice may be rendered inert by dialyzation for a short time; the inactive juice may then be restored to activity by the addition of a proper electrolyte, and, among others, they mention calcium chloride.

I have been unable to confirm Pozerski's statement by direct experiment. The diastase used by me, converted starch into sugar actively in the presence of a 1 per cent solution of calcium chloride.

[1] Delezenne: Sur le caractère brusque de l'activation du suc pancréatique par les sels de calcium, *Compt. rend. de l'acad. des sci.*, cxliv, p. 388, 1907; Sur l'activation du suc pancréatique par les sels et la spécificité du calcium, *Compt. rend. soc. biol.*, lx, p. 1070, 1906.

[2] Fernbach and Wolff: Sur le mécanisme de l'influence des acides, des bases et des sels dans la liquefaction des empois de fécule, *Compt. rend. de l'acad. des sci.*, cxl, p. 380, 1906; Étude sur la liquefaction diastasique des empois de fécule, *Ibid.*, cxlv, p. 261, 1907.

[3] Bierry and Giaja: Sur le suc pancréatique dialysé, *Compt. rend. soc. biol.*, lxii, p. 432, 1907; Sur l'amylase et la maltase du suc pancréatique, *Compt. rend. de l'acad, des sci.*, cxliii, p. 300, 1906.

Slosse and Limbosch[1] also claim that dialyzed juice is inactive. My own work along this line seems to show that diastase is exceedingly active when brought into contact with starch in distilled water. Indeed, extreme hypotonicity has impressed me as an optimal condition of the medium in which substrate and ferment meet. On the other hand, the authorities just cited prove that the activating body is an electrolyte. It follows, therefore, that the potent ferment is in loose combination with an electrolyte and that an exceedingly minute trace suffices for its proper activation.

In the absence of definite information concerning the reaction of the contents of the intestine under normal conditions, it is hardly surprising that misconceptions prevail. Thus, Bayliss and Starling[2] emphasize the high degree of alkalinity of the pancreatic juice, declaring it to have a value "equal to or greater than an $\frac{N}{10}$ sodium carbonate solution, whilst Foa[3] working with the gas chain, proved that the normal juice of a dog was equivalent to an $\frac{N}{10000}$ solution of potassium hydroxide. It is from just such apparently irreconcilable statements that conclusions must often be formulated. If preponderance be given to the evidence yielded by purely physical methods, however, then the probabilities are that the reaction of the intestinal contents is nearly neutral.

Duggan[4] was first to point out a possible relationship between the phenomena of electrolytic dissociation, and the inhibition of diastase by alkalies. It is to Wood,[5] however, that we owe the first clear statement of what is, in all probability, the true explanation. According to him: "The inhibiting action of alkalies on diastase is due to the free ions of H in case of acids and of OH

[1] Slosse and Limbosch: Note sur le dialyse des ferments digestifs en sacs de collodion, *Biochem. Centralbl.*, vii, p. 510, 1908.

[2] Bayliss and Starling: The Proteolytic Activities of the Pancreatic Juice, *Journ. of Physiol.*, xxx, p. 64, 1904.

[3] Foa: La reaction de l'urine et du suc pancréatique etudiée par la méthode electrométrique, *Compt. rend. soc. biol.*, lix, p. 867, 1905.

[4] Duggan: On the Determination of Absolute Neutrality, *Amer. Chem. Journ.*, viii, p. 211, 1886.

[5] Wood: The Action of Acids on Salts, *Amer. Chem. Journ.*, xv, p. 663, 1893; The Affinity Constants of Weak Acids and the Hydrolysis of Salts, *Ibid.*, xvi, p. 313, 1894.

in case of alkalies. In the case of salts of weak acids, it seems probable that part of the salt takes up water and dissociates into acid and base, the latter being then more fully dissociated into its ions, and the solution therefore acting like a base."

Cole [1] reached practically the same result after a very thorough investigation conducted on similar lines. The hypothesis advanced by him is interesting as a confirmation of the work of his predecessor, and also as a more detailed statement of the basic relation. He states in part: "The hydrolysis of starch by ptyalin is accelerated by the presence in solution of electronegative ions (anions) other than OH, and depressed by electropositive ions (kations), and by OH."

"The acceleration due to the negative ion varies with its nature, being greatest for anions of strong acids, and least for anions of weak acids."

"An optimal concentration of those anions can be reached. The optimal concentration is increased by increasing amounts of ferment." The ferment is destroyed by certain concentrations of hydrogen ions.

Wohlgemuth[2] places the lower limit of the inhibition of diastase at $\frac{N}{2500}$ sodium carbonate solution and $\frac{N}{5000}$ sodium hydroxide solution.

It lay near at hand, to conclude, after reasoning upon these figures, that the discrepancy between the values observed by Wohlgemuth was readily explicable by the theory of dissociation, and it occurred to me that it might be well worth while to repeat the experiments from a new point of view; seeking to determine the upper or absolute limit of diastase action, and, at the same time, the exact quantitative relation of alkali solutions which are ordinarily considered equivalent in the molecular sense. As the work progressed, its scope became gradually more circumscribed, finally centering upon the determination of the hydroxylion concentration at which diastase is powerless. I call this the critical or saturation point.

[1] Cole: Contributions to our Knowledge of the Action of Enzymes, *Journ. of Physiol.*, xxx, p. 202, 1904.

[2] Wohlgemuth: Untersuchungen über die Diastasen, *Biochem. Zeitschr.*, ix, p. 10, 1908.

The main reason for selecting the upper limit of inhibition as the point of attack was, that in so doing it would be possible to avoid maltose determinations altogether, the aim being to fix upon that particular concentration at which reduction of copper ceases. Experience has shown that this method yields striking results, the critical point being sharply defined in almost every instance.

MATERIALS AND METHOD.

Diastase from three different sources was employed in the course of my experiments.

(1) Taka-diastase, in form of a finely divided powder, was obtained from a druggist. It was preserved in a desiccator over chloride of calcium, and was weighed out with extreme accuracy when needed. The powder reduced Fehling's solution strongly when added thereto in substance, but in the proportion in which it was used—0.100 gram in 100 cc. of solution—the correction referable to this source, though definite, was small. The ferment was always thoroughly mixed with water before bringing it into contact with the substrate.

(2) My own saliva, obtained in the usual manner by chewing paraffin, was used undiluted, measured with a calibrated 1 cc. pipette.

(3) An emulsion of swine pancreas prepared by rubbing up the fresh gland in a glass mortar with distilled water, after removing as far as possible adherent fat. The mixture so obtained was then strained through fine cloth, yielding a smooth creamy fluid which showed no tendency to separate into layers on standing. It was preserved under toluol and showed no appreciable loss of activity after a considerable time. As needed, it was measured with the same pipette used for the saliva.

Accurately standardized volumetric solutions were prepared as follows: $\frac{N}{10}$ sulphuric acid, $\frac{N}{10}$ sodium hydroxide, $\frac{N}{10}$ sodium carbonate, and the corresponding $\frac{N}{100}$ dilutions.

The purest obtainable commercial starch was used, an approximate 1 per cent solution being employed in all qualitative series, and an exact 1 per cent solution, by weight, in all quantitative series.

Fehling's solution, freshly mixed by introducing 10 cc. into a 100 cc. flask and diluting with distilled water to the mark, was the test used. Each test tube received exactly 5 cc. To this amount of reagent, boiling hot, 1 cc. of the fluid to be tested was added, the mixture again brought to the boiling point and then allowed to stand.

All mixtures of ferment and substrate were made in accurately graduated 100 cc. flasks, at room temperature. In determining the critical point for a given ferment, it was the rule to prepare first, a qualitative series of flasks, in order to ascertain roughly at about what concentration an end reaction might be expected. A second series, exactly controlled by weight and volume, were then arranged in such manner as to make the end point more definite. The technique was as follows: Ten 100 cc. flasks having been made ready, and boiling distilled water being at hand, 1 gram of starch was carefully weighed into a porcelain capsule, and, after having been rubbed up to a smooth paste with a glass rod, was brought into a flask by means of a funnel, both rod and capsule being well rinsed. Boiling water was now added, the flask being shaken meanwhile to prevent the formation of lumps, and when about three-quarters filled, it was submerged in boiling water and allowed to remain until the contents were translucent. After cooling, the requisite amount of alkali was run in, a measured or weighed amount of ferment introduced, and sufficient water added to bring the contents up to the mark. Two cubic centimeters of toluol were added to each flask and the series were then kept at a temperature of 36° for periods varying from fifteen to twenty hours.

The experimental results are given in the following tables.

TABLE I.

To determine the critical point for taka-diastase in the presence respectively of decinormal sodium hydrate and sodium carbonate. One hundred milligrams of ferment acting upon 100 cc. of 1 per cent starch solution, in which the hydroxyl concentration was progressively increased. Duration, 18 hours.

$\frac{N}{10}$ NaOH cc.	1 cc. heated with 5 cc. Fehling.	$\frac{N}{10}$ Na_2CO_3 cc.	1 cc. heated with 5 cc. Fehling.
1	complete reduction	4	complete reduction
1.25	"	4.5	"
1.50	"	5	"
1.75	trace	5.5	"
2.00	"	6.0	trace
2.25	negative	6.50.....	faint trace
2.50	"	7.00.....	"
2.75	"	7.50.....	very faint trace
3.00	"	8.00.....	negative

Bearing in mind that a small correction must be made for a trace of reduction due to the ferment itself, it is evident from the table that the critical point for sodium hydrate lies not far from 1.75, whilst that for the carbonate is in the neighborhood of 6.00.

TABLE II.

To determine the critical point for saliva (ptyalin) in the presence respectively of sodium hydrate and sodium carbonate. One cubic centimeter of saliva acting upon 100 cubic centimeters of 1 per cent starch solution, with increasing hydroxyl concentrations. Duration, 15 hours.

$\frac{N}{100}$ NaOH cc.	1 cc. heated with 5 cc. Fehling.	$\frac{N}{100}$ Na_2CO_3 cc.	1 cc. heated with 5 cc. Fehling.
10	negative	10	negative
9	"	9	very faint
8	"	8	trace
7	"	7	"
6	"	6	"
5	"	5	"
4	"	4	complete reduction
3	"	3	"
2	trace	2	"
1	complete reduction	1	"

The contrast between these two series is very striking. The critical point for the $\frac{N}{100}$ sodium hydrate lying close to though a little above 1.00, whilst an end point nearly but not quite so sharply defined, showed the critical point for the other solution to lie at 4.00.

TABLE III.

To determine the critical point for an emulsion of swine pancreas in the presence respectively of sodium carbonate and sodium hydrate. One cubic centimeter of the emulsion acting upon 100 cubic centimeters of 1 per cent starch solution, with increasing hydroxyl concentration. Duration, 10 hours.

$\frac{N}{10}$ NaOH cc.	1 cc. heated with 5 cc. Fehling.	$\frac{N}{10}$ Na_2CO_3 cc.	1 cc. heated with 5 cc. Fehling.
3	complete reduction	7	complete reduction
3.25	nearly complete	7.5.....	"
3.50	trace	8	"
3.75	negative	8.5.....	"
4	"	9	"
4.25	"	9.5.....	"
4.50	"	10	"
5	"	11	negative
6	"	12	"
7	"	13	"

The value for the hydrate solution is slightly above 3; for the carbonate solution a little below 10.

It is evident from a comparison of the values exhibited in the foregoing tables, that there is a relation between the decinormal alkaline solutions other than that generally accepted. I am inclined to believe that an explanation is to be sought in the degree of dissociation taking place in the carbonate solution, and that, when finally worked out, it will be found that decinormal solutions of those alkalies, in terms of free hydroxyl, stand in relation to each other about as 1 to 3.

Although it is obviously unfair to draw conclusions from liquids as widely unrelated as human saliva and an emulsion of swine pancreas, nevertheless, it is highly significant that the latter possesses a saturation point 32 times higher than the former.

The critical point is always sharper in solutions of sodium hydrate, and the transition from the brick-red color of complete reduction to the brilliant blue of the unchanged copper solution is most striking. In case of the carbonate, the end reaction, though not as sharply defined, is easily recognized. As a rule, some copper oxide will be found in several tubes beyond that presenting the definitive change. I take it, this is indicative of some sort of reversible reaction initiated by the ferment.

The critical point is not necessarily coincident with the death of the ferment. It is possible, for example, by carefully neutralizing with dilute hydrochloric acid, to release diastase from solutions far above the inhibiting concentration. As far as I know this does not hold for the hydrate.

CONCLUSIONS.

(1) It is pointed out that diastase from three different sources maintains a constant relation to equivalent solutions of sodium carbonate and hydrate.

(2) Evidence is advanced which makes it probable that the diastase concentration of the pancreatic juice very greatly exceeds that of saliva.

(3) Diastase is shown to be a very delicate indicator of the presence of free hydroxylions.

(4) It is possible to make a sharp quantitative distinction between various fluids containing diastase in terms of decinormal sodium hydrate.

STUDIES ON ENZYMES.

II. THE DIASTATIC ENZYME OF PARAMŒCIUM IN RELATION TO THE KILLING CONCENTRATION OF COPPER SULPHATE.

By AMOS W. PETERS AND OPAL BURRES.

(*From the Zoölogical Laboratory of the University of Illinois.*)

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INTRODUCTION.

The use of copper sulphate for the efficient destruction of algæ and protozoa (but not of bacteria) in public water supplies is well known. The mode of action of such small amounts of this salt upon the cellular protoplasmic unit is an interesting pharmacological question. The main purposes of the experiments here described were, (1) to demonstrate the presence of certain enzymes in the cell bodies of paramœcia independently of bacterial contamination, and (2) to determine whether the injurious action of the copper sulphate could be explained upon the hypothesis that it inactivates enzymes of vital importance in the metabolism of the cell. Certain technical difficulties, not described in this paper, have thus far confined the experiments to the diastatic enzyme of the paramœcia.

METHOD OF RAISING CULTURES.

The cultures first used were raised in hay infusions, and seeded with material collected from the field. It was found that cultures in an infusion of wheat were more regular and certain in their development, and hence this nutrient was substituted for hay. Eight grams of ground wheat were put into about 100 cc. of water and boiled, and this was diluted to 8000 cc. with tap water. The cultures were uniformly set in this manner, making a 0.1 per cent extract of wheat. The cultures were seeded either with fresh material from the field, or with that from cultures already growing in the laboratory.

SEPARATION OF BACTERIA FROM PARAMŒCIA BY CENTRIFUGAL WASHING.

A number of animals, sufficent for the test to be made, were taken from the culture jar in a quantity of liquid which contained bacteria also, and the whole was then gently centrifuged. The culture liquid was poured off from the residual animals and the tubes filled up to the original volume with distilled water. The animals were well mixed with the water, then centrifuged again. This wash water was poured off, and the tubes were filled with distilled water for the second time, and well mixed. Then the antiseptic consisting simply of tuluol was added, 2 cc. being used to every 30 cc. of the liquid. The bacteria, being lighter than the paramœcia, were not appreciably carried down when the living material was properly centrifuged and most of them were thrown away with the culture liquid. Most of those remaining among the animals were mixed with the first wash water and poured off. The few still remaining in the material were killed by the antiseptic used, but since their number was so reduced, their presence had little or no effect on the results as the data themselves show.

DEMONSTRATION OF CATALASE AND PEROXIDASE IN PARAMŒCIA.

To demonstrate that the cell bodies of paramœcia contained catalase and peroxidase the guaiacum reaction was employed upon animals washed as previously described. Three cc. of freshly prepared tincture of guaiacum was mixed with 2 cc. of 3 per cent hydrogen peroxide in a test tube, and 1 cc. of the liquid to be tested which contained paramœcia was run in from a pipette, so that the two liquids did not mix except at the point of contact. If there was catalase present there would be an evolution of oxygen, and if peroxidase was present, the liquid would take on a blue color at the point of contact or through the whole layer. It was found that there was very little catalase and no peroxidase in the centrifugate. In the residue, however, there was an abundance of both catalase and peroxidase. The bodies of the animals turned blue, showing that the enzyme was contained in the cell, and had not been extracted.

DEMONSTRATION OF DIASTASE IN PARAMŒCIA.

To demonstrate that the cell bodies of paramœcia contained diastase, 30 cc. of liquid containing washed animals was prepared as previously described. Test and control mixtures were made, each containing 15 cc. of liquid with paramœcia in suspension, and 15 cc. of 1 per cent starch solution. To both flasks was added 1 cc. of toluol, the liquids were then agitated until all the animals were killed by the toluol, and finally the control was boiled. Both test and control were incubated at 40° C. for 24 hours. At the end of this time the sugar present was estimated by Schoorl's[1] modification of Fehling's method, which is conducted as follows:

Eight cc., i. e., two volumes of strong Fehling's solution, and 12 cc., i. e., three volumes of the solution to be tested, were put in a flask and boiled two minutes, then cooled rapidly. To this was added, in order, 8 cc. of a 10 per cent solution of potassium iodide and 8 cc. of a 15 per cent solution of sulphuric acid. If there was no starch used in the test, or if it had all been saccharified, some dilute starch solution was used as the indicator. Decinormal sodium thiosulphate was added until the blue color of the iodide of starch had disappeared. The blank test was made by using distilled water instead of the sugar solution. The number of cubic centimeters required to titrate a solution is subtracted from the blank or control value, and the weight of the sugar produced is obtained from Schoorl's table. In this experiment the values were:

Fehling's blank	10.8 cc.	$Na_2S_2O_3$.
Control	10.6 "	"
Test	7.5 "	"

The difference between the blank and the control is so small as to be negligible, but the test shows a difference of 3.3 cc. which shows that reducing substances, i. e., sugar has been produced from the starch. In Schoorl's table this corresponds to about 10.4 mg. estimated as dextrose or about 0.5 mg. per cc. of fermenting liquid. Tests made at the beginning of the incubation failed to show any appreciable amount of sugar in the quantity of material examined.

THE KILLING CONCENTRATION OF COPPER SULPHATE FOR PARAMŒCIUM.

The animals were taken from the culture jars and were washed as previously described, but no antiseptic was added. One cubic

[1] *Zeitschr. f. angew. Chem.*, pp. 633-635. 1899.

centimeter of liquid containing animals, prepared in this manner was put in a test tube and an equal volume of solution of copper of the desired concentration was then added. The tube was quickly inverted in order to mix well, then the contents were poured into a watch glass on a dissecting microscope which gave a large field at one view and which had been previously focused. The test was performed as rapidly as possible and the animals were under observation within twenty seconds from the time they came in contact with the copper sulphate. If at the first glance, the majority of the paramœcia were motionless, the concentration of copper sulphate used was regarded, not quite correctly of course, as the instantaneous killing point. In a number of tests on separate cultures, and on one culture during several days, it was found that the killing point varied from $\frac{35}{2} \times 10^{-8}$ to $\frac{60}{2} \times 10^{-8}$. In the expression $\frac{35}{2}$, the 35 is the concentration of the reagent which was used for the killing test, which of course was reduced to one-half when it became mixed with an equal volume of culture liquid in the experiment. Concentrations are expressed in gram molecules per cc.

TABLE I.

No. of cult.	Killing concentration of copper sulphate.
7–10–10–30/2	$\frac{60}{2} \times 10^{-8}$ gm. mol. per cc.
8–3–24	$\frac{35}{2} \times 10^{-8}$ " "
8–3–24/2	$\frac{40}{2} \times 10^{-8}$ " "
8–4–3/2	$\frac{45}{2} \times 10^{-8}$ " "
8–4–3/2	$\frac{51}{2} \times 10^{-8}$ " "

RELATION BETWEEN THE KILLING CONCENTRATION OF COPPER SULPHATE AND THE INACTIVATION OF DIASTASE ORIGINATING FROM PARAMŒCIA.

After having demonstrated that the cell bodies of paramœcia contain diastase, the experiments represented by the following tables Nos. II, III, IV and V were made to test the effect of copper sulphate on diastase which originated from paramœcia that had

been washed by the usual method. The starch solution was made by dissolving 1 cc. of Kahlbaum's soluble starch in water which had been heated to boiling, and then cooled somewhat before adding the starch. The copper sulphate was made in the concentration desired by diluting a stock solution, the concentration of which was 1000×10^{-8} gm. mol. per cc. After the animals had been washed the killing point was ascertained by the above described method.

The tests, as recorded in the tables, were made in 50 cc. Erlenmeyer flasks of Jena glass, and incubated at 40° C. for 24 hours. At the end of that time, the solutions were filtered through a wet filter to remove the toluol, and the amount of sugar present was ascertained by Schoorl's modification of Fehling's method which has been previously described. The volume of sodium thiosulphate used for each liquid was subtracted from the blank value, and recorded in the table.

In Table II, the flask 2 contained 10 cc. of copper sulphate of a concentration of 80×10^{-8} gm. mol. per cc. This was diluted to 4 volumes by the liquid containing the paramœcia and the starch solution, thus making the actual concentration 20×10^{-8} gm. mol. per cc., which is the same concentration as that which was found to kill immediately. In this flask there was very little if any diastatic action as shown by the small (0.4) sodium thiosulphate difference. In the other tables the concentration was lowered, in the series of tests, and in test 2 in Table IV and Table V, the actual concentration was only 5×10^{-8} gm. mol. per cc.

The fact that in Table II, the readings in 3 and 4 are lower than the blank is explained as follows. The concentration of copper sulphate used in flasks 4, 240×10^{-8} reduces 0.3 cc. of the solution of sodium thiosulphate used, and the use of this quantity as a correction would give a positive value for the sodium thiosulphate difference. Corrections of this nature might have been applied to all the tests made with copper suphate, but they were not worth while because the comparative value of the data and the conclusions drawn, would not have been affected.

All the experiments of a given table are as strictly comparable among themselves as a considerable experience in the technique used could make them. The blank value for 8 cc. of concentrated Fehling's liquid varies in the four tables, nos. II–V, but the same standardized liquid was used throughout the experiments of a single table. The tables here given represent results which were obtained after the methods had been well developed by

many preliminary and tentative experiments upon both the process of fermentation and the determination of the killing point with copper sulphate.

COMPOSITION OF A SERIES OF PARALLEL TESTS AND DATA OBTAINED.

TABLE II.

No. of flasks	1	2	3	4
Paramœcia	10 cc.	10 cc.	10 cc.	10 cc.
Starch	20 "	20 "	20 "	20 "
$CuSO_4$		10 "	10 "	10 "
		(20)	(40)	(60)
Dist. H_2O	10 "			
$Na_2S_2O_3$ diff.	4.03 cc.	0.4 cc.	− 0.15 cc.	− 0.25 cc.

Concentration of $CuSO_4$ in . . . . . 2 = $\frac{80}{4} \times 10^{-8}$ gm. mol. per cc.
" " " " . . . . . 3 = $\frac{160}{4} \times 10^{-8}$ " "
" " " " . . . . . 4 = $\frac{240}{4} \times 10^{-8}$ " "

Blank for 8 cc. conc. Fehling's solution in terms of 0.1 m. $Na_2S_2O_3$ = 9.7 cc. Killing point of paramœcia = $\frac{40}{2} \times 10^{-8}$ gm. mol. per cc. of $CuSO_4$.

TABLE III.

No. of flasks	1	2	3	4	5	Boiled. 6
Paramœcia	10 cc.	10 cc.	10 cc.	10 cc.		10 cc.
Starch	20 "	20 "	20 "	20 "	20 cc.	20 "
$CuSO_4$		10 "	10 "	10 "	10 "	
		(10)	(20)	(40)	(10)	
Dist. H_2O	10 "				10 "	10 "
$Na_2S_2O_3$ diff.	5.27 cc.	1.6 cc.	0.6 cc.	0.35 cc.	0 cc.	0.2 cc.

Concentration of $CuSO_4$ in . . . . . 2 = $\frac{40}{4} \times 10^{-8}$ gm. mol. per cc.
" " " " . . . . . 3 = $\frac{80}{4} \times 10^{-8}$ " "
" " " " . . . . . 4 = $\frac{160}{4} \times 10^{-8}$ " "
" " " " . . . . . 5 = $\frac{4}{4} \times 10^{-8}$ " "

Blank for 8 cc. of conc. Fehling's solution in terms of 0.1 m. $Na_2S_2O_3$ = 10.5 cc. Killing point = $\frac{35}{2} \times 10^{-8}$ gm. mol. per cc. of $CuSO_4$.

TABLE IV.

No. of flasks	1	2	3	4	5
Paramœcia	10 cc.	10 cc.	10 cc.	10 cc.	10 cc.
Starch, 1 per cent	20 "	20 "	20 "	20 "	20 "
$CuSO_4$		10 "	10 "	10 "	10 "
		(5)	(10)	(20)	(50)
Dist. H_2O	10 "				
$Na_2S_2O_3$ diff.	4.25 cc.	0.95 cc.	0.85 cc.	0.65 cc.	0.65 cc.

Concentration of $CuSO_4$ in..... 2 = $\frac{20}{4} \times 10^{-8}$ gm. mol. per cc.
" " " " ..... 3 = $\frac{40}{4} \times 10^{-8}$ " "
" " " " ..... 4 = $\frac{80}{4} \times 10^{-8}$ " "
" " " " ..... 5 = $\frac{200}{4} \times 10^{-8}$ " "

Blank for 8 cc. conc. Fehling's solution in terms of 0.1 m. $Na_2S_2O_3$ = 10.9 cc. Killing point = $\frac{45}{2} \times 10^{-8}$ gm. mol. per cc. of $CuSO_4$.

TABLE V.

No. of flasks....	1	2	3	4	5
Paramœcia.....	10 cc.	10 cc.	10 cc.	10 cc.	
Starch 1 per cent.	20 "	20 "	20 "	20 "	20 cc.
$CuSO_4$.....		10 "	10 "	10 "	10 "
		(5)	(10)	(30)	(5)
Dist. H_2O.....	10 "				10 "
$Na_2S_2O_3$ diff	6.95 cc.	3.35 cc.	1.85 cc.	0.15 cc.	0.15 cc.

Concentration of $CuSO_4$ in..... 2 = $\frac{20}{4} \times 10^{-8}$ gm. mol. per cc.
" " " " ..... 3 = $\frac{40}{4} \times 10^{-8}$ " "
" " " " ..... 4 = $\frac{120}{4} \times 10^{-8}$ " "
" " " " ..... 5 = $\frac{20}{4} \times 10^{-8}$ " "

Blank for 8 cc. Fehling's solution in terms of 0.1 m. $Na_2S_2O_3$ = 9.9 cc. Killing point = $\frac{51}{2} \times 10^{-8}$ gm. mol. per cc. of $CuSO_4$.

THE ENZYMIC THEORY OF THE BIOCHEMICAL ACTION OF COPPER SULPHATE AND OTHER POISONS ON LIVING PROTOPLASM.

The data of the preceding tables, nos. II, III, IV and V, show a remarkable and practically uniform correlation between the concentration of copper sulphate required to kill instantly and that concentration which markedly inhibits the action of the diastase originating from the same lot of animals. In comparing the data it should be noted that the actual concentrations expressed in 10^{-8} gm. mol. per cc., which acted upon the diastase in the mixtures described in the tables is shown by the numbers in parentheses standing in the same horizontal line with copper sulphate. The killing concentrations of copper sulphate in the four series of experiments in the order of the tables are as follows: 20×10^{-8}, 18×10^{-8}, 23×10^{-8}, 26×10^{-8}, or approximately an average of 24×10^{-8}, for the four series of experiments. It also appears throughout the tables that any concentration equal to or greater than about 20×10^{-8}, invariably and decisively depresses the activity of the ferment as compared with the No.

1 preparation of each table, which flasks contain no copper sulphate. As the concentration diminishes below 20×10^{-8}, the tables show evidence of increasing activity of the ferment although concentrations so low as 5 or 10×10^{-8}, show much injury to the enzyme. Likewise experiments made with slightly lowering concentrations of copper sulphate showed serious effects upon the animals, causing their death not instantaneously, it is true, but within the short period of a few minutes. The correlated values of 20×10^{-8} and 24×10^{-8} are to be regarded not as absolute and fixed, but as such approximations as prevail in numerical data pertaining to vital phenomena. The contration of 20×10^{-8} gm. mol, per cc. is equivalent to one gram molecule of copper sulphate pentahydrate, $CuSO_4 \cdot 5H_2O$, in five thousand liters of water or to one part by weight of this salt in twenty thousand parts by weight of water. The inactivation of the diastase by copper sulphate may be presumed to be a chemical or a physical change of the ferment for the consummation of which no extended period of time such as, e.g., 24 hours, is necessary. Experience with metallic poisons in their action upon ferments is in favor of the view that they produce their effects rather quickly when in sufficient concentrations to do perceptible injury. Therefore it may be assumed that although the incubation period was usually 24 hours, the ferment was already injured to practically the full extent possible for that concentration, at or near the beginning of the incubation period. In other words the killing effect upon the organism, and the injurious effect upon the diastase are comparable in their intensity since both were effective within the same comparatively short time. This fact leads to the consideration of the proposition whether the injurious actions of poisons like copper sulphate which act in such infinitesimally small quantities is due to the inactivation of such enzymes as are necessary in the normal metabolism of the protoplasm. We know of no facts which would lead us to ascribe so great an importance to diastase, but that the constant activity of other ferments is involved in the equilibrium of physico-chemical processes which constitute life, is highly probable in view of our present conceptions of protoplasmic processes. The quantity of copper sulphate which is instantly fatal, is too small to be due to direct quantitative

chemical action and concentration of copper sulphate in the cell bodies could scarcely occur instantly. The disturbance by the poison of a necessary catalytic agent or agents, i.e., the inactivation of indispensable and easily injured enzymes, accords much better with our knowledge of the importance and magnitude of the vital effects produced by a disproportionately small amount of material. Of course this hypothesis is not proved by the experiments above described but is rendered only possible or probable by them.

BLEACHING OF FLOUR.

By E. F. LADD and H. P. BASSETT.

(Received for publication December 15, 1908.)

The bleaching of flour or the effect of nitrous acid upon the different constituents of a flour is a subject which has been but little investigated, and is a field which promises to yield interesting results where the investigations are pushed along new lines.

During the past few years the millers of North Dakota, as well as the rest of the Northwest, if not of the entire country, have been using nitrous acid fumes or nitrogen peroxide produced in different ways for bleaching or "aging of flour." It seems that no other bleaching agent itself lends so readily to the use for this particular purpose.

Nearly all the work done along this line has been the effect on the gluten expansion, volume of loaf, blending of bleached flour with higher grades of flour, etc. With the exception of a paper submitted by Prof. J. H. Shepard, chemist of the South Dakota Experiment Station and Food Commission, nothing has been done to determine the effect of this highly poisonous gas, nitrogen peroxide, or the acids resulting therefrom on digestion. This, therefore, may be considered the most vital point to be considered because it is here that the deleterious effects, if such there are, will be produced upon man. On certain phases of this question considerable work has been done by Ladd and Stallings and by Professor Alway, whose results are published as Bulletin No. 102 of the Nebraska Experiment Station. Professor Alway, however, does not seem to have investigated the effects upon the nitrogenous constituents or to have used samples of flour bleached to the extent found upon the market in this state, and he, therefore, concludes that the use of nitrous acid is harmless since only very small quantities of the reagent are used in the bleaching of flours.

Experiments with bleached flour. Our early experiments were to determine the amount of nitrous acid or nitrites present in samples of bleached flour, and after a number of preliminary experiments with methods and samples of flour, a sample of the commercially bleached flour for trade from a North Dakota mill was found to contain:

In 5 grams, 0.0034 milligrams nitrogen.
Calculated as sodium nitrite, 0.01671 milligrams nitrogen.
In flour for one loaf of bread, 373 grams, 1.24656 milligrams nitrogen as $NaNO_2$.
This would be 6.8 parts of nitrogen as nitrite nitrogen per million.

In a sample of the clear, from the same mill, as bleached for trade, there was found:

In flour for 1 loaf of bread (373 grams), 2.4159 milligrams $NaNO_2$. This would be 12.95 parts of nitrogen as nitrite nitrogen per million.
In a sample of flour taken from a Minnesota mill there was found in the amount for one loaf, 373 grams, 1.313146 milligrams as $NaNO_2$.

Amount in bread. A sample of bread, produced from the flour as commercially bleached from a North Dakota mill, was analyzed to determine the amount of nitrites present, when calculated as sodium nitrite. Three hundred and seventy-three grams produced a loaf weighing, approximately, 18 ounces or 509 grams, and was found to contain 0.44352 milligrams as sodium nitrite, or approximately, one-third of the amount originally found in the flour.

A sample of bread produced in the same way from over-bleached flour, from a North Dakota mill, showed 3.546 milligrams sodium nitrite, or, slightly less than one-third of that found in the flour.

Effect of bleaching on fat. Fat extracted from the unbleached North Dakota mill flour, that had stood in the laboratory for nine months, gave iodine number (Hanus Mod.) 101.2°. A sample of the same flour, bleached, and which had stood in the laboratory for nine months after bleaching, gave iodine number (Hanus Mod.) 84.1°. Other tests showed similar results. The oil from the bleached sample was more stringy and like the results of the action of the nitrous acid or nitric acid, or the combined action of the same in producing elaiden.

Nitrogen combined with oil. Two samples of flour, the one bleached and the other unbleached of the same, were extracted with ether. About 200 grams of flour were used, the ether distilled off, leaving the fat, and the residue taken up in redistilled petroleum ether solution, carefully filtered, and the petroleum ether thoroughly evaporated off, thus getting rid of any impurities which may have been originally in the oil from ether extrac tion.

The tests for the presence of combined nitrogen and fat were made as follows:

A piece of well cleaned metallic sodium was strongly heated in an ignition tube and three drops of the wheat flour oil were then allowed to fall upon the partly vaporized sodium. When cool, the contents of the tube were first treated with a little alcohol and then with water. This solution was then filtered, treated with a few drops of sodium hydroxide solution and with ferrous sulfate solution, then boiled for a minute or two. Just enough of dilute hydrochloric acid was added to dissolve the precipitate and, finally, a drop or two of ferric chloride solution was added; the presence of nitrogen being indicated by a precipitate of Prussian blue.

Oil from unbleached flour, no nitrogen.
Oil from bleached flour, considerable combined nitrogen.

Numerous other tests made upon the oil from bleached and unbleached flour gave similar results.

Tests were next made upon the oil extracted from bread produced from bleached and unbleached flour without the addition of any foreign fat. The loaf was dried, ground to fine meal, extracted with ether, and purified with petroleum ether, as previously described, with results as follows:

Oil from bread, unbleached, no combined nitrogen.
Oil from bread, bleached, combined nitrogen.

The amount of nitrogen in the oil from the sample of bread made from the bleached flour was less than in that of the flour from which the bread was produced.

Nitrous and nitric acid present. Tests were made showing the presence of both nitrous and nitric acid, or nitrites and nitrates, as reacting material in flour, which had been bleached by the use

of nitrogen dioxide or tetroxide. In many of the experiments made, from one-third to one-half of the amount of nitrites found in the flour are recovered in the bread made by the usual process with yeast as a leavening agent. In the case of biscuit, rolls and like products where baking powder or soda is used and in the thinner products, the nitrites are in a large measure destroyed by the oxidation to nitrates.

The effect of bleaching on digestion. In Professor Shepard's work he investigates the effect of nitrous acid on the action of the digestive enzymes on pure corn starch. This, however, leaves out the action on flour, bread and other products where protein bodies, and in some cases, starch are present, and on this point the following work is reported.

The effect of bleaching on digestion of gluten and bread: The pepsin solution used in these experiments was prepared according to the directions given in Simon's Physiological Chemistry, p. 450, which is as follows:

> The following three solutions were prepared:
>
> (A) To 294 cc. of water 6 cc. of hydrochloric acid (1.20 sp. gr. diluted to 10) was added.
>
> (B) In 100 cc. of solution (A) 0.06 gr. of pepsin was dissolved.
>
> (C) 90 cc. of solution (A) was brought to 40° C. in a digestion oven and 10 cc. of solution (B) was then added.
>
> These experiments were carried out in the following manner:
>
> A test tube three-fourths inch in diameter was cut off forming a short tube about 3½ inches long: in the bottom of this a hole was blown to allow the digestion solution to come in contact with the substance to be digested. In the bottom of this tube was placed a plug of glass wool packed with a glass rod, and on this plug was placed the gluten or bread to be digested. This was then immersed in the flask containing the digestion solution, and the time noted until the bread or gluten had disappeared. The time could not be accurately determined, but when there was a marked difference, as is shown in some cases, a relative figure could be determined.

Eight trials were made in the manner described using the raw gluten obtained by washing out 10 grams of commercially bleached flour by the usual method and treating with 200 cc. of solution (C). The following results were obtained:

GLUTEN FROM BLEACHED FLOUR.

No. of tube.		Time of digestion. Hours.
1	.....	8
2	.....	8½
3	.....	9
4	Left over night and the oven cooled down.	
5	Started 7:30 a.m. and allowed to run until 6 p.m. and could hardly be said to be fully digested.	
6	.....	9
7	.....	10
8	.....	8

Ten trials were made under the same conditions as in the case above, using the gluten from 10 grams of unbleached flour, with the following results:

GLUTEN FROM UNBLEACHED FLOUR.

No. of tube.		Time of digestion. Hours.
1	.....	4
2	.....	4½
3	.....	4
4	.....	4
5	.....	5
6	.....	4½
7	.....	5½
8	.....	6
9	.....	6
10	.....	6

In the above cases raw gluten was used, and raw material of this character is much harder to digest than when cooked in some form, so it was thought advisable to try the action of the digestion fluids on baked gluten.

The baked gluten from 10 grams of flour was treated in the manner previously described, but no such marked difference could be detected as the digestion was much more rapid and the difference in time could not be noted as well.

Baked gluten from bleached flour was first tried with the following results:

No. of tube.		Time of digestion. Hours.
1	.....	3
2	.....	3½
3	.....	2½
4	.....	2½
5	.....	3½
6	.....	3½

It will be noted that two of the above trials required only 2½ hours, but in most cases over 3 hours were required.

Baked gluten from unbleached flour was next tried in the same manner with the following results:

No. of tube.	Time of digestion. Hours.
1	2
2	1¾
3	2
4	2½
5	2

In only one case did the baked gluten from unbleached flour require over two hours.

The next trials along this line were carried out on bread made from each of the flours. The following table gives the results obtained with bread made from bleached flour:

No. of tube.	Time of digestion. Hours.
1	2
2	1¾
3	2

The results with the bread made from the unbleached flour are given below:

No. of tube.	Time of digestion. Hours.
1	2
2	1½
3	1¾

It will be noted that the time of digestion was in favor of the unbleached flour, but the times of digestion are so close that the results are necessarily not as definite as in the case of raw gluten.

Pancreatic digestion. The next problem was to investigate the action of pancreatic digestion on the three different products. The pancreatic solution was prepared for this work according to the directions given by Simon, which are as follows:

To 100 cc. of water 0.28 grams of pancreatin and 1.5 grams of sodium bicarbonate were added. Two hundred cubic centimeters of this solution was used in each experiment.

Four experiments on pancreatic digestion were made, using in each case the raw gluten obtained from 10 grams of flour. The following results were obtained:

PANCREATIC DIGESTION OF RAW GLUTEN FROM COMMERCIALLY BLEACHED FLOUR.

No. of tube.	Time of digestion. Hours.
1	3
2	3½
3	3¼
4	3

The following results were obtained for the unbleached flour:

No. of tube.	Time of digestion. Hours.
1	1½
2	2
3	1¾
4	2
5	2

It will be noted that in the case of the bleached flour 3 hours or more were required for the complete digestion, while the unbleached digested in 2 hours or less.

Several tests of each case were made with baked gluten, but the difference, however, could not be so well established as the rate of digestion was so rapid that the difference in time was not as marked as with raw gluten.

BAKED GLUTEN FROM COMMERCIALLY BLEACHED FLOUR.

No. of tube.	Time of digestion. Hours.
1	¾
2	1
3	¾

BAKED GLUTEN FROM UNBLEACHED FLOUR.

No. of tube.	Time of digestion. Hours.
1	½
2	¾
3	¾

It will be noted that in every experiment in both cases the gluten digested in one hour or less.

The bread from bleached and unbleached flour was next tried, with the following results:

BREAD FROM BLEACHED FLOUR.

No. of tube.	Time of digestion. Hours.
1	1¾
2	1¾
3	2
4	2
5	2

BREAD FROM UNBLEACHED FLOUR.

No. of tube.	Time of digestion. Hours.
1	1¼
2	1
3	1
4	1
5	1½

By accident a much better test of digestion for the bread was discovered; that of digestion by means of the ordinary mold. Some of the bread used in the preceding experiments was allowed to stand in sealed Mason jars, and it was noticed that a large formation of mold appeared on the bread from unbleached flour in four days, while that from the commercially bleached flour remained free from any appreciable amount of mold for nearly ten days. However, the jars containing these samples of bread were not sterilized, and it was thought that this formation of mold might possibly be due to different conditions existing in the different jars. To determine this point the following experiments were carried out, in which we tried to make the conditions about the same.

Jars were sterilized in the hot-air oven at 115° C. and used in experiments which were carried out as follows:

First Jar. Bread made from unbleached flour was inoculated with some of the mold on the unbleached bread used in the above experiment.

Second Jar. Bread made from bleached flour was introduced and inoculated with some of the same mold used in no. 1.

Third Jar. Bread made from unbleached flour was introduced and inoculated with some of the mold on the bleached bread used in the former experiment.

Fourth Jar. Bread made from bleached flour was introduced and inoculated with the same mold as in no. 3.

The reasons for making these two distinct tests in carrying out these experiments with the mold was due to the appearance of

the mold on the bleached bread, as it seemed to be of a more destructive nature and it was thought that possibly it might be that it was of a different character. The results from the above experiments were as follows:

The bread from the unbleached flour, in both cases, stood in the jars tightly sealed for two days before the mold made any appreciable show. The bread, however, from the commercially bleached flour, in both cases, resisted the action of the mold for five days.

The samples, however, were allowed to stand for 10 days and further development noticed. The bread made from unbleached flour, at the end of this time, was almost entirely eaten up; while that from the bleached flour was well covered with the mold, but the texture remained good.

The tests with the mold indicate the same general conclusions as those brought out by the peptic and pancreatic digestions, as they show that the mold digests the bread with a marked difference.

The point might be raised that bleaching was a good thing as it acted as a preservative for the bread, but the pure food law wisely does not allow preservatives of other character, such as sulfites in meats, to be used, and this, no doubt, is similar in character so far as its effect is concerned.

The point was now brought up, if the nitric oxide acted upon the flour, what portions did it act upon—the starch, the gluten, or, only on the fat? To determine this point the following observations were made:

If the nitric oxide acted upon the starch it would, no doubt, form a very low nitrated nitro-starch product, and if this were the case, when treated with an acid, and heated up, would give off nitric oxide that would act upon starch, iodide paper, or a solution of starch and potassium iodide; but, when 100 grams of flour were treated in this manner, no such reaction was observed, showing, without doubt, that there could not be any action on the starch. However, it was noticed that a large amount of gas was given off, more than could be accounted for by being dissolved in the acid solution or by the expansion of the small amount of air left in the flask. This phenomenon was not noticed when unbleached flour was used.

The next problem now was to determine what this gas was and, as we had no action in the starch, it remained either to be a decomposition product from fat or gluten.

A 50-gram sample of bleached flour was then taken and the fat extracted. This fat was then treated in the same manner as the flour, but no such action was noticed; thus, it could only be caused from an action on the gluten, but what this action was remained to be determined.

In studying over the reactions which might occur between such bodies, as the protein bodies, and nitric oxide, we will readily see the possibility of a diazo reaction, and if this occurred the gas should be nitrogen. A Shiff's azotometer was arranged to collect such gas, and 100 grams of flour were treated in the manner described above. After the complete apparatus had been filled with carbon dioxide to drive out all the air, the acid, which had been previously boiled and was still warm, was introduced through a separatory funnel, and the gases formed driven over into the azotometer by more carbon dioxide (the carbon dioxide being absorbed by the potassium hydrate in the azotometer) the nitrogen collecting in the top over the potassium hydrate. A very small amount of the nitrogen was given off but enough to be readily measured. This, however, if carried farther, would, no doubt, give means of determining the amount of nitrogen dioxide that actually went into the chemical combination; and, if the flour was over-bleached, the excess could then be approximately determined.

Further evidence to prove this point has recently been secured. In bleaching some flour to the limit to try to duplicate that which collects on the agitators and in other parts of the bleacher, it was also noticed that quite an appreciable heat was developed, indicating, no doubt, that a definite chemical reaction was going on. The flour also absorbed the first volume of nitric oxide with extreme readiness. Further, it was noticed that there was a point when this readiness of absorption by the flour was at an end and in order to make the flour further take up more of the gas, vigorous shaking was required and the stream of gas had to be diminished.

This would seem to indicate that the gas acted on the gluten or some part of the gluten until its affinity for the gas was satis-

fied and the further action was either a secondary action on the gluten or that a low nitrated nitro-starch was formed: this point is now under investigation.

The next work relating to this subject was done in the bleaching of different flours. A first patent durum flour was bleached with varying amounts of the nitric oxide under the same conditions, using 5 cc., 10 cc., etc., to the 100 grams of flour. The one bleached with 20 cc., however, was slightly overbleached. This blended with the best white hard wheat flour was used for a standard. The following mixture being made:

Blended. Per cent.	Standard. Per cent.	Remarks.
10	90	Could not be detected by slick.
20	80	" " " "
40	60	" " " "
30	70	" " " "

It is evident that by the ordinary tests 30 per cent of durum could be introduced without detection. The same thing was tried with flour which was bleached, with 10 cc. nitrogen dioxide to 100 grams, with practically the same results.

CONCLUSIONS.

As the result of the experiments given in the preceding pages, and in previous articles, we may summarize the facts in the following conclusions:

(1) That nitrous and nitric acid are two of the constituents formed from the bleaching of flour with nitrogen peroxide.

(2) The nitrites and nitrates, or nitrite and nitrate reacting material, are among the products formed in the flour.

(3) That bread as baked in the home by the domestic method will contain from one-third to one-half of the nitrite reacting material found in the flour.

(4) Oil properly extracted and purified from unbleached patent flour contains no nitrogen.

(5) Oil extracted from bleached flour and purified by the same methods gives a strong reaction for nitrogen, thus, confirming the statement made by Lewkowitsch.

(6) Oils from unbleached flours have an iodine absorption number (Hanus' method) of 101 or more, while the iodine absorp-

tion number for oils from bleached flours, when properly purified, will have a lower iodine number in proportion to the amount of bleaching.

(7) The difference in the iodine number and the difference in the nitrogen content of the oils show that the bleaching agent has acted upon the fat of the flour.

(8) Flours aged for nine months showed no reduction in iodine number, while the same flour bleached and aged for the same length of time showed a reduction of 17.1 points, indicating that the artificial bleaching is not the same as the natural aging of flours.

(9) The proportion of nitrates in the bread increases as the nitrites decrease.

(10) The method of baking will determine to what extent the nitrites are changed or eliminated in the bread.

(11) Artificial digestion experiments with pepsin solutions showed that the gluten from the unbleached flour was digested in 4 hours and 57 minutes; while, under the same conditions the gluten from the bleached flour was digested in 8 hours and 40 minutes.

(12) The baked gluten from the bleached and unbleached flours showed similar variations but not so wide, the time of digestion being much less; the same is true for the bread made from such flours.

(13) In pancreatic digestion the glutens digested in 3.19 hours from bleached flour, and in 2.31 hours from unbleached flour. The time of digestion in pancreatic solutions of the baked gluten and of the bread was in favor of the unbleached product.

(14) The experiments made with the keeping quality of bread made from bleached and unbleached flour demonstrated the antiseptic effect of the bleaching agent.

(15) It has been demonstrated that there is an action upon the gluten, apparently a diazo reaction. The bleaching agent acted upon the gluten of the flour changing its composition so that nitrogen gas was given off when the flour was treated with an acid.

(16) The fact that the xanthoproteic reaction takes place demonstrates further that the bleaching agent has acted upon the gluten or protein of the flour.

ON THE COMPOSITION OF DILUTE RENAL EXCRETIONS.

By A. B. MACALLUM and C. C. BENSON.

(*From the Physiological Laboratory of the University of Toronto.*)

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The problem of the factors in the formation of urine is as yet not near a solution which may be generally accepted. The discussion of the question in the literature of the subject still centers chiefly around the two theories, the one of Bowman-Heidenhain, the other of Ludwig. That discussion some years ago appeared to be in effect closed with the publication of Heidenhain's contribution[1] to it, fortified as it was by the observations of Nussbaum[2] on the phenomena of secretion in the kidney of the frog which seemed to indicate very clearly that while the reflected epithelium of Bowman's capsule in the glomerulus is chiefly concerned in the elimination of water from the blood and lymph, the cells of the renal tubules remove the urea and related organic constituents of the urine. Indeed Heidenhain's own investigation on the excretion of sodium sulphindigotate in the kidney of the dog also decidedly postulated an excretory function on the part of certain portions of the renal tubule, a property not at all provided for in Ludwig's theory.

Ludwig's theory has again been more recently advanced and defended, not indeed in the original form in every respect, by Starling,[3] Cushny,[4] Henderson and others and this has led to a

[1] Versuche über den Vorgang der Harnabsonderung, *Arch. f. d. ges. Physiol.*, ix, p. 26, 1875; Also, Die Harnabsonderung, Hermann's *Handbuch der Physiologie*, *v*, p. 279, 1883.

[2] Ueber die Secretion der Niere, *Arch. f. d. ges. Physiol.*, xvi, p. 139, 1878; Fortgesetzte Untersuchungen über die Secretion der Niere, *Ibid.*, xvii, p. 580, 1879.

[3] The Glomerular Functions of the Kidney, *Journ. of Physiol.*, xxiv, p. 317, 1899.

[4] On the Diuresis and the Permeability of Renal Cells, *Journ. of Physiol.*, xxvii, p. 429, 1902; On Saline Diuresis, *Ibid.*, xxviii, p. 431, 1902.

series of investigations on the subject, but though the balance of the evidence advanced by these is against the filtration-reabsorption theory it is not decisive. In not a few of these investigations the kidneys under examination were subjected to abnormal conditions, such as certainly may be brought about by the intravenous injection of strong solutions of salts. Like quantities of such salts do not under any circumstances obtain in the blood of the normal animal, not even when such solutions are injected into the intestinal cavity and it can scarcely be held that either the glomerular or the tubular epithelium remains considerably unaffected when brought into contact with plasma or lymph containing such unusual quantities of salts or such unusual constituents. It is not at all, of course, to be contended that the results obtained from such investigations are not of value, for their importance is very great, and eventually, when the facts decisive regarding the processes of urine formation have been ascertained, their place and their significance in the summing up will be recognized.

A question of fundamental import in the discussion is whether filtration obtains at all in the normal kidney. If its occurrence could be demonstrated the cardinal principle in the Ludwig theory would be sustained and another point would inevitably follow, for to explain the differences in composition between the filtrate from the blood plasma and lymph on the one hand, and on the other, the urine as it leaves the kidney, involves of necessity reabsorption of some of the constituents, the inorganic at least, by the tubular cells. It may of course be objected that the differences might be caused by the tubular epithelium adding by secretion to the filtrate as it passed along the lumina of the tubules, an addition not to be excluded in view of the results obtained by Bainbridge and Beddard[1] and Cullis[2] in the case of the renal tubules of the frog. In answer to this it may be said that to postulate filtration in the glomeruli and secretion in the tubular epithelium of such a character as to eliminate from the blood large quantities of salts is not a consistent position to

[1] Secretion by the Renal Tubules in the Frog, *Biochem. Journ.*, i, p. 255, 1906.

[2] On the Secretion in the Frog's Kidney, *Journ. of Physiol.*, xxxiv, p. 250, 1906.

assume, especially in view of the fact that the tubular epithelium must be supposed in this case to contribute at times much more of the inorganic constituents than are filtered off by the glomeruli. What intellectually is to be gained from the assumption of such a position?

The determination of the question whether filtration occurs is one of great difficulty and it has been hitherto attempted only indirectly. Filtration would involve variations in amount of the urine with alterations in blood pressure and consequently efforts have been directed to ascertain if a correlation exists between blood pressure and the quantity of urine formed. These have shown that there is a connection between the two, and that when the pressure in the capillaries sinks below 30 mm. of mercury the formation of urine ceases, but it has not yet been demonstrated that there is a proportional relation between the fluid eliminated and the blood pressure. Nor has the attack on the problem by a study of the pressure in the renal tubules and in the pelvis of the kidney been more successful. A filtration pressure in the capillaries of the glomeruli would involve a head of pressure in each tubule immediately adjacent to its glomerulus sufficient at least to overcome the resistance offered by the tubules themselves. Now high pressure exerted in the pelvis of the kidney through its ureter does cause the cessation of the formation of urine and moderate degrees of pressure should, at least, diminish its quantity if filtration is a factor but, curiously, as Schwarz,[1] Brodie and Cullis[2] in the dog and Steyrer[3] in the human subject have determined, a certain degree of pressure in the pelvis of one kidney increases the quantity of the urine formed as compared with that derived at the same time and under the same blood pressure from the other kidney. This makes it evident that if filtration does obtain in the glomeruli, its occurrence is masked by the operation of other forces which may be grouped under the term "secretory."

There is one way along which as yet the solution of the question has not been attempted and that is by the analyses of normal

[1] *Centralbl. f. Physiol.*, xvi, p. 281, 1902.

[2] On the Secretion of Urine, *Journ. of Physiol.*, xxxiv, p. 224, 1905.

[3] Ueber osmotische Analyse des Harns, Hofmeister's *Beiträge*, ii, p. 330, 1902.

urines of the greatest possible attenuation. In certain conditions of diuresis the urine formed is so great in volume and so rapidly produced that the swiftness of the current along the secreting as well as along the collecting portions of the renal tubules would permit of very little reabsorption and at the same time the additions made by the secreting portions would be very slight. The more dilute such urines are the more they would approximate to a filtration of the kind demanded by the Ludwig theory even when the concentration in the salts is much less than it is in the blood plasma or lymph, for then at least there should be a parallelism in relative proportions between the inorganic elements in the blood plasma on the one hand and the same elements in such dilute urines.

The quantities of sodium, potassium, calcium, magnesium and chlorine in human plasma have not, since Schmidt's[1] time (1850), been accurately ascertained and his results are defective owing to the methods of determination then employed, but the estimations of the quantities of those elements in the blood plasma of mammals have been made by Aberhalden,[2] who has found that in the serum of the horse Na = 0.3291 per cent; K = 0.02183 per cent; Ca = 0.00795 per cent; Mg = 0.0027 per cent, and Cl = 0.3726 per cent, and taking this as a basis of calculations of proportions there ought to be present in the plasma for every 100 parts by weight of sodium:

K, 6.63; Ca, 2.41; Mg, 0.82; Cl, 113.22

and if filtration occurs in the formation of these dilute urines, then in the latter there should be a like or nearly similar series of proportions in the same elements.

Such dilute urines have been obtained but have never been analyzed. Dreser[3] found that after copious draughts of Munich beer the concentration sank so low that the Δ was $-0.16°$. Engel and Scharl[4] found in the case of an epileptic patient

[1] *Charakteristik der epidemischen Cholera gegenüber verwandten Transudationsanomalien. Eine physiologische-chemische Untersuchung*, Leipzig und Mitau, 1850.

[2] Zur quantitative vergleichenden Analyse des Blutes, *Zeitschr. f. physiol. Chem.*, xxv, p. 65, 1898.

[3] *Arch. f. exp. Path. u. Pharm.*, xxix, p. 302, 1892.

[4] *Zeitschr. f. klin. Med.*, lx, p. 225, 1906.

who had taken 1400 cc. of Salvator water the minimum Δ was $-0.10°$ C. This compared with the Δ of the blood serum of the human subject, which is $-0.56°$, indicates the degree of dilution which such urines manifest. The capacity of the kidneys to form urines of such low concentrations is by no means rare and it has been found in individuals who did not at all suspect it to be present. All that suffices to test its occurrence in any particular case is to ingest large quantities of distilled water, 1–3 liters, in a couple of hours and in not a few individuals it will be followed by the formation of large amounts of urine of very low concentrations. In one case the senior author obtained early in this investigation 205 cc. of a 10 minute period of which the Δ was $-0.075°$. In such a case the elimination of water by each kidney was not less than 10 cc. per minute and it is not surprising that the Δ rose to such a height. In other cases it was found that it was readily possible to obtain after draughts of large quantities of distilled water urines in which the Δ ranged from $-0.09°$ to $-0.60°$, which latter is a little lower than that of blood plasma.

The possibility of obtaining readily such dilute urines for examination led the authors to attempt to determine how high the Δ in such urines may be raised. It was supposed that if a urine whose Δ was $-0.05°$ were obtained its analysis might reveal unique features which would help in the solution of the question of urine formation but the uppermost limit of $-0.075°$ was never exceeded and it was obtained only once while it was a matter of no particular difficulty to obtain fluids whose Δ ranged from -0.09 to $-0.15°$.

The failure to reduce the concentrations to the low limit aimed at, led to the attempt to determine quantitatively the inorganic constituents in such dilute urines, but owing to the small quantities (65 to 200 cc.) passed during each period of 10 minutes it was not possible to make complete analyses. The calcium and magnesium in such fluids were under the amounts which would have made it possible to determine them quantitatively. By adding together the fluids from several consecutive periods of 10 minutes each it might have been ascertained how much calcium and magnesium was eliminated in the sum of those periods, but this would not have been of any service, for each

urine formed during those periods had very often a different Δ and as we subsequently learned there was a variation in the composition from period to period even in cases in which the Δ was the same and consequently it was held to be of no object to obtain such estimations.

The chief inorganic constituents in such urines are the salts of sodium and potassium of which the most abundant are their chlorides. There is no difficulty in determining the amount of chlorine and of the potassium, but that of sodium, accurately ascertained, present in such dilution and in such small quantities of the urines, offers insuperable difficulties. It can only be determined indirectly or differentially, that is, either by converting all the bases into sulphates (of sodium, potassium, calcium and magnesium, estimating directly the potassium, calcium and magnesium and finally the sodium by difference, or by first of all removing all the calcium and magnesium salts and converting the sodium and potassium salts into their chlorides which may be weighed and, having directly determined the potassium, the sodium may then be ascertained.

With the quantities of fluid at the authors' disposal it is obvious that the estimation of the sodium in such urines could not be serviceably performed and in consequence only the determinations of the potassium and the chlorine were carried out.

It was felt, however, that even this much would be of value and the results obtained have justified this view.

The chlorine was estimated gravimetrically, as the volumetric method owing to uncertainty as to the exact end point, introduces so large an error when dealing with small quantities of very dilute solutions of chlorides. Indeed when the amount of chlorine in a solution is from 0.02 to 0.03 per cent and one uses for estimation volumetrically from 20 to 30 grams of the fluid the error may well be between 5 and 8 per cent. In consequence the gravimetric method alone was employed. A decinormal solution of nitrate of silver containing 1.5 per cent of nitric acid was added to a carefully weighed quantity, usually about or near 10 grams of concentrated urine, but to between 20 and 50 grams of the dilute excretion, and the mixture placed in the dark for 24 hours, when it was carefully filtered through two thicknesses of an "ashless" filter specially adapted to hold

back fine precipitates. The precipitate was washed on the double filter seven or eight times with distilled water in order to remove even traces of the adherent nitrate compound and when on drying the precipitate, as was the case with dilute urines, could not be detached from the filter, both filter and precipitate were incinerated together, which of course, reduced a large proportion of the silver chloride to the metallic silver. The residue was in consequence carefully treated with nitric acid to dissolve the metallic silver, the nitrate formed as carefully converted into the chloride, after which the usual method of procedure was followed.

The general method of estimating the potassium in urine is to remove by precipitation the sulphuric and phosphoric acids of the sulphates and phosphates and the magnesium and calcium of their compound (including traces of iron), and then on evaporation of the filtrate to dryness to get rid of the ammonium salts and the organic compounds by heating to a dull red heat in a platinum capsule, after all which the sodium and the potassium salts only remain and these can be converted into chlorides and weighed, the chloride of potassium then being isolated as the double salt of potassium and platinum. Even when the quantity of the urine is large and the concentration of its inorganic constituents sufficient to enable the investigator to reduce the limits of error in the result, the method is tedious, but when at the most 50 to 75 cc. only of a very dilute urine are available for such a purpose the method is not only tedious but inapplicable, for the quantities of calicum and magnesium salts and of the sulphuric acid and phosphoric acid are too minute to be effectually removed by precipitation and they then remain to influence very considerably and, of course, unfavorably the exact determination of the potassium and the sodium.

The method of estimating the potassium which meets all the difficulties is that known as Finkener's[1] and consists in the conversion of all the bases into sulphates which are then dissolved, with some hydrochloric acid, in water to which more than a sufficient quantity of platinum chloride in solution is added to

[1] Poggendorff's *Annalen*, cxxix, p. 637; also Rose, *Handbuch der anal. Chemie*, 6 Aufl. by Finkener, ii, p. 923.

combine with all the potassium present. The mixture is then carefully evaporated in a porcelain capsule on a water bath down to the volume of 1 to 3 cc., the platinum salt uncombined with potassium is thoroughly extracted with alcohol and ether and the potassium determined from the weight of the reduced platinum filtered off from the residue.

This method was considerably improved by Dittmar in his investigations on the composition of the sea water of the Challender Expedition and it was also used with some modifications by the senior author in his studies on the inorganic composition of the Medusæ[1] and in the case of a number of trial estimations of potassium in solutions of accurately known strength of this element, found to give on the average 99.4 per cent of the potassium present. Even when phosphates were present with the sulphates the determination of the potassium was not materially affected, so that this obviates the necessity of getting rid, as in the other methods, not only of the sulphuric, but also of the phosphoric acid. More recently he has employed it in the determination of the potassium present in a wide range of physiological fluids and has ascertained that it is the most time-saving as well as the most accurate method of estimating potassium present in such fluids.

The method, as the authors employed it in this case, was as follows. A quantity of the urine, not more than 10 grams of the concentrated, but from 16 to 60 grams of the dilute urines, was accurately weighed out in a platinum capsule, evaporated to dryness in it and the residue carefully heated in a fume cupboard with an amount of sulphuric acid varying with the concentration of the urine from 1 to 5 cc. This destroyed the organic matter and after the uncombined sulphuric acid was expelled the residue was subjected for several minutes to a degree of heat which gave a red glow to the capsule and its contents. This removed by volatilization all the ammonium sulphate present. Care was taken not to expel any of the potassium sulphate by raising the heat to too high a degree. The residue, which consisted of sulphates and some phosphates, was

[1] On the Inorganic Composition of the Medusæ, *Aurelia flavidula* and *Cyanea arctica*, *Journ. of Physiol.*, xxix, p. 213, 1903.

then dissolved in distilled water free from all traces of ammonia, and the solution collected in a porcelain evaporating dish. To it were added 2 to 5 cc. of hydrochloric acid along with considerably more of a 10 per cent solution of platinum chloride than sufficed to combine with all the potassium present and the mixture, with frequent stirring of the contents, was slowly evaporated on a water bath till its volume did not exceed 2 cc. On this 40 cc. of absolute alcohol were poured and the mixture was allowed to stand for two hours under a bell jar, when 20 cc. of ether were also added. At the end of two hours more the greater part of the ether-alcohol solution was carefully removed by decantation, a fresh mixture (40 cc. of alcohol and 20 cc. of ether) was added and allowed to extract for an hour when a similar decantation was employed. The addition of a fresh mixture followed later by decantation was made usually five times, with the result that the only platinum salt in the residue was that combined with the potassium.

The residue after being cautiously dried was heated in the evaporating dish to 300° C. for half an hour in a current of dry hydrogen conveyed by an inverted funnel into the dish. In order to provide for a complete reduction of all the platinum present to a metallic condition the residue on cooling was dissolved in a few cubic centimeters of water. The solution with the contained reduced platinum was evaporated to complete dryness and the residue once more reduced in dry hydrogen gas at 300° C.

The residue was now treated with hot water and filtered through two filters of the "ashless" type, specially adapted to remove very fine precipitates. The employment of such filters is necessary since there is a tendency of the reduced platinum to form particles approaching in size those of a colloidal suspension of this element.

The filters with the reduced platinum were, after being dried, incinerated and as the ash of two such filters weighed not more than 0.00008 gram the whole of the incinerated residue was regarded for the purposes of the determination of the potassium as reduced platinum and the weight of the latter multiplied by 0.40195 gave the weight of the potassium. The value 0.40195 is the theoretical one based on the atomic weight of platinum being

194.8 and on that of potassium being 39.15. Though, as stated earlier, only 99.4 per cent of the platinum is on the average obtained in such reductions and, consequently, a slightly higher factor than 0.40195 might be employed, it was thought best to employ throughout the theoretical value.

In not a few of the cases the amount of fluid at our disposal did not permit us, unfortunately, to make duplicate analyses. In all these, however, the greatest care was taken to avoid error and where there was the slightest suspicion of its occurrence the whole of the filtrate from the reduced platinum, containing all the potassium previously combined with the latter, was concentrated with fresh quantities of platinum chloride and hydrochloric acid and the potassium reëstimated through the reduced platinum so obtained. In the majority of these the duplicates agreed completely with the original; but more instructive than these are those where the differences between the duplicates and the original determinations were the greatest as in two of the estimations for the periods 13, 14, and 15 of Series I, which gave reduced platinum in the following amounts:

	No. 13.	No. 14.	No. 15.
Original estimation.....	0.0102	0.0163	0.0127
Duplicate estimation from filtrate of original.....	0.0102	0.0161	0.0129

From these results it is reasonable to assume that where duplicate estimations could not be made the results obtained were fairly accurate and this assumption is strengthened when one examines the results in the three series, for in these the potassium determined in the successive ten minute periods shows a remarkable gradation in amount from period to period.

Early in the investigation the authors merely attempted to ascertain if in the urines of the greatest degree of dilution the chlorine and the potassium were present in the ratio in which they obtain in the blood plasma or in the lymph. According to Schmidt's estimations already referred to, the ratio should be 100 : 9.10; but Lehmann[1] averaging from his own and Schmidt's determinations got values for the two elements from which the ratio 100 : 8.86 may be obtained. These give a too high value

[1] Lehmann's *Physiological Chemistry*, London, 1851, ii, p. 160.

due to the fact that the method of determining the potassium then in use was not sufficiently accurate. Vierordt[1] gives the mean of four series of analyses of Wanach,[2] two of Schmidt and two of Arronet[3] with results which give for the chlorine and the potassium a ratio of 100 : 6.83. This is very near the correct point for the senior author found that the ratio in hydrocele lymph to be 100 : 6.97. Accordingly if any of the dilute urines were merely filtrates the proportion of the chlorine to the potassium should be 100 : 6.97, but in the very first one examined, that whose Δ was $-0.075°$ C., the ratio was 100 : 35.71, that is, the potassium was relatively more than five times as abundant as it is in lymph, or in blood plasma.

It was subsequently found also that the ratio in the passage from the concentrated urines to the very dilute is not a constant one and in consequence it was thought best to ascertain how this obtains. The results of this line of work are to be found in Tables I, II and III embodying the analyses of three series of dilute urines.

In these it is to be noted that in no single instance is the proportion even approximately that found in lymph or plasma and there is a still greater discrepancy in some of the more dilute urines. In Series I, No. 1, with $\Delta = 0.73°$ C. the ratio is 100 : 32, but in No. 6 whose Δ was $-0.11°$ C. the ratio was 100 : 67.99, that is, more than double.

The analyses of such dilute urines, therefore, not only do not support the filtration theory but tell directly against it while they furnish facts which are of great significance as regards the secretion theory.

It has been shown by Nasse,[4] Denis[5] and Leichtenstern[6] that

[1] *Anatomische, physiologische und physikalische Daten und Tabellen*, p. 196, 1906.

[2] Quantitative Analyse des Menschenblutes. Dissertation, Dorpat, 1887, Reference by Vierordt.

[3] Ueber die Menge und Vertheilung des Kaliums, Natriums und Chlors im Menschenblut. Dissertation, Dorpat (St. Petersburg), 1888, Ref. by Vierordt.

[4] Wagner's *Handwörterbuch der Physiologie*, i, p. 128, 1842.

[5] *Essai sur l'application de la chimie à l'étude physiologique du sang de l'homme et à l'étude physiologico-pathologique, hygiénique et thérapeutique des maladies de cette humeur*, Paris, 1838.

[6] *Untersuchungen über den Haemoglobingehalt des Blutes* etc., Leipzig, Vogel, 1878.

SERIES I.

No.	Time of collection.	Quantity collected in grams.	Δ	Cl.	K.	Ratio of K to Cl (Cl = 100).
				per cent.	*per cent.*	
1	1.40	280	−0.73	0.203	0.065	32.02
2	2.00	252	−0.13	0.03042	0.02208	72.58
3	2.10	180	−0.13	0.03306	0.0238	71.99
4	2.20	120	−0.15	0.03296	0.02099	63.68
5	2.30	94	−0.14	0.02963	0.02107	71.11
6	2.40	89	−0.11	0.02987	0.02031	67.99
7	3.00	151	−0.115	0.02786	0.01790	64.26
8	3.10	100	−0.11	0.02775	0.01621	58.05
9	3.20	88	−0.11	0.02757	0.01616	58.61
10	3.30	93	−0.11	0.02698	0.01728	64.04
11	3.40	93	−0.12	0.02694	0.01684	62.51
12	3.50	101	−0.11	0.02829		
13	4.00	76	−0.11	0.02797	0.01588	56.77
14	4.10	107	−0.09	0.03024	0.01805	59.35
15	4.20	100	−0.11	0.02848	0.01711	60.07
		1924				

SERIES II.

Quantity of distilled water taken = 2500 cc.

No.	Time.	Quantity collected in grams.	Δ	Cl.	K.	Ratio of K to Cl (Cl = 100).
				per cent.	*per cent.*	
1	1.10	382	−1.935	0.8019	0.3184	39.70
2	2.45	160	−1.39	0.5222	0.31838	60.96
3	3.00	94	−0.385	0.11545	0.05828	50.48
4	3.15	126	−0.235	0.0694	0.03334	48.04
5	3.30	211	−0.245	0.06466	0.02495	38.58
6	3.40	139	−0.205	0.05745	0.02171	37.78
7	3.50	137	−0.235	0.05808	0.02375	40.89
8	4.00	156	−0.22	0.05958	0.02433	40.83
9	4.12	167	−0.21	0.06358	0.02821	44.36
10	4.22	130	−0.21	0.06457	0.03293	50.99
11	4.33	131	−0.205	0.06337	0.03802	59.99
12	4.43	109	−0.185	0.06321	0.03179	50.29
13	4.53	125	−0.165	0.04724	0.02529	53.53
14	5.05	113	−0.18	0.05069	0.01928	38.04
15	5.15	119	−0.20	0.04946	0.01537	31.08
16	5.25	130	−0.305	0.04955	0.01494	30.14
		2429				

SERIES III.

Quantity of distilled water taken = 2000 cc,

No.	Time.	Quantity collected.	Δ	Cl.	K.	Ratio of K to Cl (Cl = 100).
				per cent.	*per cent.*	
1	12.45		−1.36	0.6457	0.2336	36.17
2	3.00	136	−1.00	0.3842	0.18206	47.38
3	3.20		−0.18	0.02291	0.0117	51.07
4	3.30	75	−0.185	0.02402	0.009965	41.48
5	3.42	91	−0.145	0.02527	0.007406	29.30
6	3.50	130	−0.135	0.02696	0.008945	33.17
7	4.02	168	−0.095	0.02347	0.007544	32.14
8	4.10	111	−0.15	0.02814	0.007955	28.26
9	4.20	129	−0.185	0.03491	0.00967	27.69
10	4.33	137	−0.115	0.02103	0.008416	40.02
11	4.53		−0.205	0.02137	0.009106	42.60
12	5.13	218	−0.13	0.02308	0.010796	46.77
13	5.33	211	−0.17	0.02630	0.01433	54.48
14	6.53	226		0.02718	0.02052	75.53
15	7.40	261		0.03173	0.02463	77.62
		1892				

drinking large quantities of water does not appreciably alter the volume of the blood. Magendie[1] did not find the specific gravity of the blood to be altered thereby and Leichtenstern, using the photometric method, did not obtain any evidence of change in the concentration of the hæmoglobin in the blood even in individuals who drank as much as seven liters of water daily.

More recently Engel and Scharl[2] found by the refractometric method that no decrease in the concentration of the blood occurs when water is copiously ingested and that even an increase in concentration obtained when the kidneys were exceedingly active, secreting a very dilute urine with a Δ of −0.16° or −0.15° C. The conclusion to be drawn is that the water is not retained in the blood but is removed very quickly. Our own observations tend to confirm this. In an individual who drinks as much as two liters in as many hours practically no diminution in the number of red corpuscles may be demonstrable with the hæmocy-

[1] Quoted by Dreser, *loc. cit.*

[2] *Zeitschr. f. klin. Med.*, lx, p. 225, 1906.

tometer, which ought not to be the case if the ingested water increases the volume of the blood. It is, of course, to be admitted that even with the greatest precautions taken in the use of the hæmocytometer (of the Thomas-Zeiss form) two observers may vary very considerably in the determination of the number of the corpuscles present in each cubic millimeter, but this should not exceed 2 per cent, yet in the estimation of the corpuscles in the blood of the individual before and after the ingestion of a large quantity of water the difference may be less than this. In one of the two cases in which we made estimations of the number of corpuscles, the latter, immediately before the ingestion of water began, were 4,732,000 per cubic millimeter. After two and one-half hours when 2700 cc. had been ingested and 1260 cc. excreted by the kidneys the corpuscles numbered 4,638,000 per cubic millimeter. In the second case, immediately before the ingestion of water the blood as estimated contained 4,820,000 corpuscles per cubic millimeter, but two and one-half hours later, when the kidneys were secreting vigorously, the estimate was 4,900,000. If, as is doubtful, the first case is to be taken as indicating the degree of dilution which the blood was made to undergo by the ingestion of large quantities of water, an addition of water to the blood to the extent of about 2 per cent of its volume stimulated the kidneys to an activity, in the removal of water, twenty to twenty-four times that ordinarily exerted.

It may, of course, be postulated that a portion of the water removed from the blood goes into the lymph, which circulating in the lymph channels of the glomeruli and about the renal tubules promotes filtration, but such a decrease in the concentration of lymph in the kidney derived only from the blood driven through the kidney could not possibly reach the degree demanded to account for the removal of water on the filtration theory. It must also be pointed out that in the renal glomeruli the endothelial walls of the capillaries and the reflected epithelium of Bowman's capsule are in such intimate contact as at times to suggest that they form a syncytial structure, in which there could be no provision for capillary channels. Indeed the structure of the glomerulus would seem to indicate very decidedly that in its elimination of urinary constituents, the lymph plays no recognizable part.

The only explanation which embraces all the facts is that the removal of water is the result of a true secretory process which may be enhanced by such a small excess of water in the blood plasma as may escape detection, and that the act of secreting water is independent of that involved in the secretion of other constituents of urine. In no other way is it possible to account for the formation of urine of such low concentrations as we have obtained and analyzed.

As regards the two constituents, the chlorine and the potassium, there is a separate rate of excretion for each, as may be seen on examination of the tables. When the urine is passing from the concentrated to the dilute condition the chlorine excreted quickly diminishes but the potassium does not keep pace and may even for time, as in Nos. 1 and 2, Table II, remain constant, but eventually its diminution begins and is finally reaches its minimum later than does the chlorine. It is this lagging in the activity, a *hysteresis* as it were, in the glomerular membrane as regards the elimination of potassium compared with that of chlorine that increases the ratio of the former to the latter in urines of some of the periods and decreases it in others.

This hysteresis is shown in periods Nos. 1 to 8, Table II, and in Nos. 1 to 4, Table III, as well as in Table I in which, however, the initial value of $\frac{K}{Cl}$ is not regained even approximately in the later periods. It is worthy of note that in Nos. 6 to 11, Table II, the value of potassium is rising while the amount of the chlorine is almost stationary but, later in Nos. 13 to 15, the quantity of potassium is falling while the amount of the chlorine does not show any important variation.

It is not possible to account for these results except on the assumption that the membrane or membranes engaged in the elimination of the chlorine and the potassium vary in activity for each element independently. As the chlorine is chiefly if not wholly combined as chlorides of sodium and potassium it is obvious that the rate of excretion of sodium chloride is different from that of potassium chloride, being greater when the urine is diminishing in concentration than it is in the later periods of dilution. This differential activity may be supposed also to

obtain in the case of the phosphates, sulphate and carbonates of sodium, potassium, magnesium, calcium and ammonium and this would explain Cushny's results, who found that the excretion of the chlorides, phosphates and sulphates did not run parallel and accounted for it by postulating that the chlorides are more readily absorbed by the epithelial cells of the renal tubules than are the phosphates and sulphates.

This differential rate of excretion would account for the variations in the chlorides and sulphates that appear during the stages of activity in the kidney of the dog as ascertained by Brodie and Cullis. They found that after the intravenous injection of quantities of sodium chloride and sodium sulphate, the rate of elimination of each salt was different and in the later stages the chloride diminished and even disappeared from the urine though the diuresis was still at its height.

In saline diuresis the presence of large quantities of such compounds as the sulphate of sodium and even an excess of chloride of sodium in the membrane, influences sometimes favorably, sometimes unfavorably the rate of elimination of the other salts.

A differential secretory activity was observed by Wohlwill[1] in the urine of a young patient with orthostatic albuminuria. During the orthostatic periods $\frac{K}{Cl}$ was more than double what it was in the urine secreted during the recumbent position but the acidity decreased. In a case of cardiac insufficiency the same conditions were found. Wohlwill, however, explains the results he found by postulating that the chlorine and the potassium are not eliminated by the same secretory mechanisms in the renal tubules and glomeruli.

It is probable that the normal differential activity is the result of an adaption on the part of the excreting membrane, developed through long ages in contact with a circulating fluid of more or less fixed composition. This differential activity would essentially depend on the solubilities of certain plasma salts in the excreting membrane itself and these again on the physical constitution of the membrane, but the latter as a sensitive living structure may alter with the composition of the fluid in

[1] *Arch. f. exp. Path. u. Pharm.*, liv, p. 389, 1906.

contact with it, and consequently the differential activity may also vary, with the result that the rate of elimination of those constituents of the plasma which are in excess of the normal may be enhanced. It is only in this way that we can explain the almost constant retention in the blood plasma of proportions in the amount of the sodium, potassium, calcium and magnesium, such as those already referred to and which without important variations obtain throughout mammals. Renal excretion is, on this view, fundamentally a matter of solubility in the excreting membrane and as the solubility must vary for different salts from time to time, differential activity obtains.

SUMMARY.

1. In urines of low concentration obtained through the ingestion of large quantities of distilled water and whose Δ ranges from $-0.30°$ to $-0.075°$ the value of $\frac{K}{Cl}$ is never that which obtains in the blood plasma and is usually much greater than that which obtains in the concentrated urine formed immediately before the experiment began.

2. This increase in the value of $\frac{K}{Cl}$ in dilute urines is due to a "lag" in the diminution of the secretion of the potassium as compared with the chloride during the decrease in the concentration. This lagging behind, or "hysteresis" may be found again, though not always, when the urine begins to increase in concentration, the value of $\frac{K}{Cl}$ then falling because the potassium slowly and the chlorine (especially of sodium chloride) rapidly increases. In some cases, notably towards the end of a series, the rate of the excretion of the potassium may, relatively to the chlorine, rapidly increase or rapidly decrease.

3. The elimination of water is not due to filtration but to the physiological activity of the renal membranes involved in the elimination.

4. The removal of potassium salts and of chlorides from the blood by the kidneys is not due to filtration but to forces which may be termed "secretory," that is, it is caused by an activity which

is apparently selective, or differential, but which may be explained as due to differences in solubility of the different inorganic constituents of the plasma in the secreting membrane. The solubility, either relatively, or absolutely, or both relatively and absolutely, would be altered by changes in the constitution of the membrane brought about by the action on it of unusual constituents of the plasma or of constituents of unusual proportions.

ON THE DEPRESSION OF THE FREEZING POINT OF WATER DUE TO DISSOLVED CASEINATES.

By T. BRAILSFORD ROBERTSON and THEO. C. BURNETT.

(From the Rudolph Spreckels Physiological Laboratory of the University of California,)

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I. INTRODUCTORY.

The question whether or not the proteins possess, in solution, a definite osmotic pressure has been the subject of much controversy. The original investigations of Graham[1] appeared to indicate that colloids in general exert a high osmotic pressure. Subsequent investigators, however, attributed these results to an admixture of crystalloids. Thus Sebanejew[2] found that the depression of the freezing point which is brought about by egg-albumen could be almost entirely accounted for by the estimated pressure of the inorganic constituents of the protein, and Sebanejew, and Alexandrow[3] estimated from their determinations that the molecular weight of egg-albumen must be at least 14000 in order to account for the extremely slight lowering of the freezing point which could be attributed to the protein alone; the proteoses Sebanejew estimated, also from cryoscopic determinations, to possess a molecular weight of from 2000 to 3000, while the molecular weight of the peptones was estimated to be 400 or less.[4] Tamman[5] measured the difference between the lowering of the freezing point of the serum of the horse before and after coagulating the proteins by heat, and removing them, and found that the difference amounted to only .006°, the experimental error of

[1] Graham: *Phil. Trans. Roy. Soc.*, London, cli, p. 183, 1861.

[2] Sebanejew: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 87, 1890; xxiv, p. 558, 1891.

[3] Sebanejew and Alexandrew: *Journ. of the Russian Phys.-Chem. Soc.*, p. 7, 1891, quoted after Maly's *Jahresber. f. Tierchem.*, xxi, p. 11, 1891.

[4] Sebanejew: *Ber. d. deutsch. chem. Gesellsch.*, xxvi, p. 385, 1893.

[5] Tamman: *Zeitschr. f. physikal. Chem.*, xx, p. 180, 1896.

the cryoscopic method being about .005°. Ludeking found that even 40 per cent of gelatin in solution did not perceptibly alter the boiling point,[1] and Krafft and Wiglow[2] confirmed his results. Dreser[3] and Koeppe[4] both found that the removal of protein from solutions had no measurable effect upon the osmotic pressure of the solution. Bugarsky and Liebermann[5] estimated the depression of the freezing point due to dissolved egg-albumen, albumose and pepsin, and their ash, separately, and deducted the latter from the former. They estimated in this manner the molecular weight of egg-albumen to be 6400, that of albumose to be 2400, and that of pepsin to be 760. They also found that if egg-albumen, albumose or pepsin be added to solutions of acid or alkali, the cryoscopic depression of the resultant solution is less than the sum of the depressions due to the acid or alkali and the protein dissolved separately; while if the egg-albumen, albumose or pepsin were dissolved in salt solutions, the cryoscopic depression of the mixture was found to be (within the limits of experimental error) identical with the sum of the cryoscopic depressions due to the protein and the salt dissolved separately. Bugarsky and Tangle[6] carried out an extended series of investigations aiming at the determination of the cryoscopic depression due to the non-electrolytes of the blood, among which they inc uded the proteins. They determined the chlorine content of the blood, and from that, deduced the equivalent molecular concentration of the sodium chloride in the blood and its conductivity; they then measured the conductivity of the blood, and subtracting from it that due to sodium chloride, considered the remainder as due to sodium carbonate, and estimated therefrom the molecular concentration of the sodium carbonate. They then subtracted the cryoscopic depression due to the sodium chloride and the sodium carbonate contents thus estimated, from the observed cryoscopic depression of the blood; the difference they ascribed to the non-electrolytes and proteins. In this way they estimated the con-

[1] Ludeking: *Annalen der Physik und Chem.*, xxxv, p. 552, 1888.
[2] Krafft und Wiglow: *Ber. d. deutsch. chem. Gesellsch.*, p. 2566, 1895.
[3] Dreser: *Arch. f. exp. Path. u. Pharm.*, xxix, p. 314, 1896.
[4] Koeppe: *Arch. f. d. ges. Physiol.*, lxii, p. 571, 1896.
[5] Bugarsky and Liebermann: *Ibid.*, lxxii, p. 51, 1898.
[6] Bugarsky and Tangle: *Ibid.*, lxxii, p. 531, 1898.

centration of non-electrolytes in horse's blood to be about .056 mol. per liter. The accuracy of this determination is, however, very questionable since, as Laqueur and Sackur,[1] Sackur,[2] Hardy,[3] Robertson[4] and others have shown, the proteins, when combined with acids and bases, play a very appreciable part in the transportation of electricity through their solutions; hence it is not admissible to regard the conductivity of the blood as being due to inorganic constituents alone to the exclusion of the proteins.

Starling has endeavored to measure directly the osmotic pressure of the proteins in blood serum by using for his osmometer a membrane permeable to salts, but impermeable to proteins;[5] he estimated in this way the osmotic pressure of the proteins in blood serum to be about 40 mm. of mercury, corresponding to a lowering of the freezing point of less than .005° and to an average molecular weight of the serum protein of the order of 25000. Reid, on the contrary, finds that proteins purified by repeated crystallization, re-solution and recrystallization, frequently possess, in solution, no measurable osmotic pressure, and he concludes that provided every precaution be taken to exclude impurities (among which he includes inorganic constituents) from the protein solution it will invariably be found to possess no osmotic pressure whatever, and that the osmotic pressures observed in solutions of incompletely purified proteins are due, not to the protein, but to the associated impurities.[6]

It appears to us that many of the above observations and conclusions are vitiated by the fundamentally erroneous conception that the inorganic constituents which are found associated with proteins are invariably present as impurities, and not in a state of chemical combination. The manner in which this assumption vitiates conclusions regarding the molecular weight (estimated

[1] Laqueur and Sackur: *Beitr. z. chem. Physiol. und Pathol.*, iii, p. 196, 1903.

[2] Sackur: *Zeitschr. f. physikal. Chem.*, xli, p. 672, 1902.

[3] W. B. Hardy: *Journ. of Physiol.*, xxxiii, p. 251, 1905.

[4] T. Brailsford Robertson: *Journ. of Physical Chem.*, xi, pp. 437 and 542, 1907; xii, p. 473, 1908.

[5] Starling: *Journ. of Physiol.*, xviii, p. 312, 1896; xxiv, p. 317, 1899.

[6] Reid: *Journ. of Physiol.*, xxxi, p. 438, 1904.

from the depression of the freezing point or directly from the osmotic pressure) of proteins will be clear from the following considerations. Bases and acids have been demonstrated to form definite salts of a constant composition with casein,[1] serum globulin[2] and protamin,[3] and there can be no doubt whatever that similar compounds are formed with other proteins. In solutions of casein and of serum globulin it can be shown that as the neutral point is approached the alkali-binding power becomes less[4] and from conductivity and other data it can be shown that this phenomenon is due to a polymerization of the protein molecule, according to equations of the type, $HXOH + HXOH = HXXOH + H_2O$,[5] so that at or in the neighborhood of the neutral point, molecular aggregates are formed of such dimensions that, in the cases of proteins mentioned, the solution assumes the character of a suspension and the protein is precipitated; addition of acid or alkali shifts the equilibrium in the direction of lower complexes and the protein goes into solution again in the form of a salt.[6] Similar phenomena may be safely assumed to occur in other protein solutions, although the polymerization of the protein which occurs when the uncombined protein is set free may not result in actual precipitation. The elaborate precautions which have been taken by many observers to free the protein under investigation from accompanying inorganic substances,

[1] Söldner: *Landwirt. Versuchstat*, xxxv, p. 351, 1888: quoted after Van Slyke and Hart, *Amer. Chem. Journ.* xxxiii, p. 461, 1905; Laqueur and Sackur: *Beitr. z. chem. Physiol. und Pathol.* iii, p. 196, 1903; Van Slyke and Hart: *Amer. Chem. Journ.*, xxxiii, p. 461, 1905; T. Brailsford Robertson: this *Journal*, ii, p. 317, 1907.

[2] W. B. Hardy: *Journ. of Physiol.*, xxxiii, p. 251, 1905. T. Brailsford Robertson: *Journ. Physical Chem.*, xi, p. 437, 1907.

[3] Kossel: *Zeitschr. f. physiol. Chem.*, xxv, p. 165, 1898. Taylor: *Univ. of Calif. Publ.*, 1, p. 7, 1904.

[4] Thus at neutrality to phenolphthalein, 1 gm. of casein neutralizes 8 cc. of $\frac{N}{10}$ alkali, while at neutrality to litmus it neutralizes only 5 cc. Cf. Van Slyke and Hart, *loc. cit.*, and T. Brailsford Robertson, *loc cit.*

[5] T. Brailsford Robertson: *Journ. of Physical Chem.*, xii, p. 473, 1908. Cf. also T. Brailsford Robertson: this *Journal*, v, p. 147, 1908.

[6] One may compare the behavior of the proteins with that of acetic acid, which combines with bases as CH_3COOH, but, when uncombined with bases forms, in solution, double molecules possessing the formula $(CH_3COOH)_2$.

have, therefore, defeated their own ends, by converting the protein into aggregates so enormous as to possess a necessarily immeasurably small osmotic pressure.

Since it appears probable therefore, that the dissolved *salts* of proteins may exert a measurable osmotic pressure in solution and hence cause an appreciable lowering of the freezing point of water in which they are dissolved, we have undertaken a series of determinations of the lowering of the freezing point of water which is brought about by dissolved (neutral) caseinates.

II. EXPERIMENTAL.

It has been shown by Soldner, Laqueur and Sackur, Van Slyke and Hart, Robertson and others,[1] that casein combines with bases in equivalent molecular proportions to form solutions neutral to litmus such that 1 gram of casein equals 5 cc. of $\frac{N}{10}$ alkali, and to form solutions neutral to phenolphthalein such that 1 gram of casein equals 8 cc. of $\frac{N}{10}$ alkali. It has, furthermore, been shown by Robertson[2] that when alkaline solutions are "saturated" with casein, the amount of casein which dissolves is exactly that necessary to form the "neutral" caseinate (neutral to litmus) of the base and that solutions so prepared are neutral to litmus.

Determinations were made of the freezing points of solutions of various concentrations of both the "neutral" and the "basic" (neutral to phenolphthalein) caseinates. The solutions of the "neutral" caseinates were made up as follows: alkali of a given concentration was shaken up with about 20 per cent excess of casein until no more casein would dissolve (one-half to three-quarters hour) and the solution was then filtered through rapid filtering paper. The cryoscopic depression was determined in the usual way.[3] The following were the results obtained.

[1] *Loc. cit.*

[2] T. Brailsford Robertson: this *Journal*, ii, p. 317, 1907.

[3] Excessive cooling was carefully avoided—the undercooling never being more than about 3°. The solutions were inoculated with a minute crystal of ice to facilitate freezing. The casein used was that prepared by Eimer and Amend, purified according to the method described by Robertson: this *Journal*, ii, p. 317, 1907.

TABLE I.

NEUTRAL CASEINATES.

(*Experimental error of determination, + .0025°.*)

BASE.	CONCENTRATION OF BASE "SATURATED" WITH CASEIN.	Δ	INDICATING A CONCENTRATION OF:
NH_4OH	$\frac{M}{50}$	0.045	$\frac{M}{41}$
"	$\frac{M}{50}$	0.035	$\frac{M}{58}$
"	$\frac{M}{33.3}$	0.055	$\frac{M}{34}$
"	$\frac{M}{33.3}$	0.055	$\frac{M}{34}$
"	$\frac{M}{20}$	0.07	$\frac{M}{26}$
"	$\frac{M}{15}$	0.095	$\frac{M}{19}$
KOH	$\frac{M}{50}$	0.0325	$\frac{M}{57}$
"	$\frac{M}{50}$	0.0375	$\frac{M}{49}$
"	$\frac{M}{33.3}$	0.0425	$\frac{M}{44}$
"	$\frac{M}{33.3}$	0.0475	$\frac{M}{39}$
"	$\frac{M}{20}$	0.05	$\frac{M}{37}$
"	$\frac{M}{20}$	0.075	$\frac{M}{23}$
"	$\frac{M}{15}$	0.10	$\frac{M}{18.5}$
LiOH	$\frac{M}{59.5}$	0.03	$\frac{M}{62}$
"	$\frac{M}{40}$	0.045	$\frac{M}{41}$
"	$\frac{M}{24}$	0.07	$\frac{M}{26}$
"	$\frac{M}{18}$	0.08	$\frac{M}{20}$
$Ca(OH)_2$	$\frac{M}{91}$	0.015	$\frac{M}{120}$
"	$\frac{M}{91}$	0.015	$\frac{M}{120}$
"	$\frac{M}{91}$	0.0175	$\frac{M}{101}$
"	$\frac{M}{61}$	0.02	$\frac{M}{92.5}$
"	$\frac{M}{61}$	0.02	$\frac{M}{92.5}$
"	$\frac{M}{61}$	0.02	$\frac{M}{92.5}$
"	$\frac{M}{45.5}$	0.025	$\frac{M}{74}$
"	$\frac{M}{45.5}$	0.025	$\frac{M}{74}$
"	$\frac{M}{45.5}$	0.025	$\frac{M}{74}$

The solutions of the "basic" caseinates were made up in two different ways. In the preliminary experiments the amount of casein necessary to form the "basic" caseinate of the alkali was weighed out and added to the solution, which was then carefully stirred until all the casein had dissolved. It is always trouble-

some, however, on account of foaming, to ascertain with certainty when the casein has completely dissolved; consequently, in later experiments, excess of casein was shaken up with alkali until approximate "saturation," and then an aliquot part of the filtered solution was titrated against the alkali with phenolphthalein and the additional amount of alkali necessary to form the "basic" caseinate, thus ascertained, was added to the bulk of the solution, and this solution was diluted to the various concentrations employed. The following are the results obtained, those obtained with solutions made up by the earlier method being indicated in this table by an asterisk. It will be seen that the results obtained by the two methods are in satisfactory harmony.

TABLE II.

Basic Caseinates.

(*Experimental error of determination* ± *0.0025°.*)

BASE.	CONCENTRATION OF BASE "SATURATED" WITH CASEIN.	Δ	INDICATING A CONCENTRATION OF:
NH_4OH	$\frac{M}{36}$	0.04	$\frac{M}{46}$
"	$\frac{M}{34}$	0.04	$\frac{M}{46}$
"	$\frac{M}{27}$	0.0475	$\frac{M}{39}$
"	$\frac{M}{25}$	0.05	$\frac{M}{37}$
* "	$\frac{M}{20}$	0.07	$\frac{M}{26.5}$
"	$\frac{M}{18}$	0.055	$\frac{M}{34}$
"	$\frac{M}{17}$	0.075	$\frac{M}{25}$
* "	$\frac{M}{15}$	0.10	$\frac{M}{18.5}$
*KOH	$\frac{M}{50}$	0.035	$\frac{M}{53}$
* "	$\frac{M}{33}$	0.04	$\frac{M}{46}$
"	$\frac{M}{35}$	0.055	$\frac{M}{34}$
"	$\frac{M}{25}$	0.06	$\frac{M}{31}$
* "	$\frac{M}{20}$	0.07	$\frac{M}{26.5}$
* "	$\frac{M}{17}$	0.0875	$\frac{M}{20}$
"	$\frac{M}{15}$	0.095	$\frac{M}{19}$
LiOH	$\frac{M}{40}$	0.04	$\frac{M}{46}$
"	$\frac{M}{30}$	0.05	$\frac{M}{37}$
"	$\frac{M}{20}$	0.07	$\frac{M}{26}$
$Ca(OH)_2$	$\frac{M}{91}$	0.015	$\frac{M}{120}$
"	$\frac{M}{68}$	0.02	$\frac{M}{92.5}$
"	$\frac{M}{45.5}$	0.0225	$\frac{M}{60}$

III. THEORETICAL.

From the results which we have obtained it is evident that the caseinates of the alkalies and the alkaline earths bring about a definite and easily measurable depression of the freezing point of water in which they are dissolved, and hence we may conclude that in all likelihood they also exert a definite osmotic pressure. It will be observed that the depression which is brought about is in every case very nearly that which would be brought about by a solution of the same molecular concentration as that of the alkali which is neutralized by the casein (provided electrolytic dissociation is negligible) and hence (neglecting electrolytic dissociation) we may conclude that each *molecule* of the alkali gives rise, upon combination with casein, to *one molecule* of the caseinate; and hence that casein, in these solutions, behaves towards bases essentially as a monobasic acid, as Robertson[1] has previously concluded from conductivity data.

If, however, casein were behaving in these solutions as a pluribasic acid, a considerable degree of dissociation might account for the effects observed. Thus:

A salt of a dibasic acid	and a monacid base,			50	per cent	dissociated
or "	tribasic	"	"	66	"	"
or "	tetrabasic	"	"	75	"	"
or "	pentabasic	"	"	80	"	"

would in solution, yield a cryoscopic depression approximately equal to that of a solution of the same concentration of a nearly undissociated salt of a monobasic acid and a monacid base. Since, however, the solution of the di-acid base, $Ca(OH)_2$, neutralized to phenolphthalein or litmus by casein yields a cryoscopic depression of the same magnitude that is yielded by an equimolecular solution of potassium hydrate similarly treated with casein,[2] we should have to assume, were we to regard casein, as, for example, a dibasic acid, that *calcium caseinate* s not dissociated at all, while *potassium* or *ammonium* caseinate is dissociated 50 per

[1] T. Brailsford Robertson: *Journ. of Physical Chem.*, xi, p. 542, 1907; xii, p. 473, 1908.

[2] In the more dilute solutions; in the more concentrated solutions there is considerable discrepancy, as will be observed by referring to the tables. The reason for this cannot at present be assigned.

cent.[1] Hence were we to regard casein as behaving in these solutions as a dibasic acid, we should expect to find solutions of the caseinates of the alkalies excellent conductors of electricity, and those of the caseinates of the alkaline earths very poor conductors. No such profound difference between the conductivities of these solutions is, however, found. Thus, the conductivity of a 0.01 M solution of ammonium hydrate "saturated" with casein, is 508×10^{-6} reciprocal ohms, while that of a .009 M solution of calcium hydrate "saturated" with casein, is 266×10^{-6} reciprocal ohms, indicating a difference in the degree of dissociation, of the two solutions, it is true, but not a difference so great as we should be led to anticipate from our freezing point determinations, were casein a dibasic acid.

We may therefore regard these results as confirming the view that casein, in solutions neutral to litmus or to phenolphthalein, behaves towards bases essentially as a monobasic acid. Our results also confirm the view expressed by Robertson[2] that casein undergoes polymerization as the neutral point is approached. Thus a $\frac{M}{80}$ solution of potassium hydrate rendered neutral to phenolphthalein by the addition of casein, depresses the freezing point .035°, corresponding to a $\frac{M}{83}$ solution; upon addition of casein in the proportion to that already in solution of 8 to 5, yielding a solution of the "neutral" caseinate, neutral to litmus, the depression is still approximately that corresponding to a $\frac{M}{50}$ solution. Hence, although the quantity of casein in the solution is increased by over 50 per cent, yet the number of molecules of caseinates remains the same; in other words the casein in combination with bases undergoes polymerization as the neutral point is approached.

[1] To illustrate this reasoning by a numerical example, if a 1-molecular solution of a monacid base neutralized by a dibasic acid were 50 per cent dissociated, the depression of the freezing point would be that of a 1-molecular solution. A 1-molecular solution of a di-acid base neutralized by a dibasic acid, also 50 per cent dissociated, would yield a depression of the freezing point corresponding to a 1.5 molecular solution; but were it *undissociated* it would yield a depression of the freezing point exactly equal to that of the 50 per cent dissociated salt of the dibasic acid with a monacid base, namely that corresponding to a 1-molecular solution.

[2] T. Brailsford Robertson: *Journ. of Physical Chem.*, xii, p. 473, 1908.

Finally, our results indicate an average molecular weight approximating to 2000 for casein combined with bases in neutral solution, and to 1400 for casein combined with bases in solutions neutral to phenolphthalein; thus agreeing satisfactorily with the results obtained by other methods.[1]

IV. CONCLUSIONS.

(1) The "neutral" (neutral to litmus) and "basic" (neutral to phenolphthalein) caseinates of potassium, lithium, ammonium and calcium, when dissolved in water, depress the freezing point; and this depression is definite in amount and readily measurable.

(2) The results are such as to indicate that casein behaves towards bases essentially as a monobasic acid, possessing, when combined with bases in solutions neutral to phenolphthalein, a molecular weight of approximately 1400; and when combined with bases in solutions neutral to litmus, a molecular weight of approximately 2000.

[1] T. Brailsford Robertson: *loc. cit.*; cf. also the remarks of Emil Fischer, *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 1754, 1907, concerning the probable molecular weights of proteins.

THE CEREBROSPINAL FLUID IN CERTAIN FORMS OF INSANITY, WITH SPECIAL REFERENCE TO THE CONTENT OF POTASSIUM.[1]

By VICTOR C. MYERS.

(From the Laboratory of the Connecticut Hospital for the Insane.)

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The researches of the pathologist have given us little assistance in explaining the various forms of insanity; and chemical investigations have been even less successful in throwing light upon this difficult problem. Of the various modes of studying the pathological changes which probably take place in the deranged central nervous system, the chemical examination of the cerebrospinal fluid is one which would appear worthy of consideration. Since the brain and spinal cord are both bathed with the fluid, it would seem possible that the metabolism in these organs might also bring about changes in the composition of their fluid environment. Furthermore, the ease with which the cerebrospinal fluid is obtainable by lumbar puncture, either during life or after death, greatly facilitates such an investigation.

On account of the large amount of lecithin in the nervous system, it would seem very probable that in cases of nervous degeneration, appreciable quantities of the base cholin might be set free. The question has frequently been investigated, especially in acute degenerative insanities such as dementia paralytica.

[1] The author is especially indebted to Dr. Jessie W. Fisher, Pathologist in charge of this laboratory, for her many kindnesses during the investigation. A more detailed clinical study, including the cytology, bacteriology, etc., of the cerebrospinal fluid referred to in this paper, will subsequently be published by Dr. Fisher. The author also desires to express his thanks to Drs. A. C. Thomas and L. R. Brown of the Hospital Staff for their kindness in making the lumbar punctures during life; likewise to Dr. Henry S. Noble, superintendent of this institution, without whose willing coöperation this investigation would have been impossible.

Mott and Halliburton[1] first claimed to have demonstrated the presence of cholin, their work being corroborated later by the researches of Gumprecht,[2] Donath,[3] Coriat,[4] Rosenheim,[5] and others. These investigators all reported cholin to be present in acute degenerative insanities. Gumprecht stated that if sufficient fluid were employed, he was able to detect cholin in the normal cerebrospinal fluid, and that it was increased not only in dementia paralytica, but in many other diseases of the nervous system, especially in meningitis. Donath found cholin present in the cerebrospinal fluid in epilepsy, though not in quite as large quantity as in paresis; and he considered it an important factor in the production of epileptic convulsions. Rosenheim was able to detect cholin in certain cases of nervous degeneration, but found that it was absent where the disease was "functional" or quiescent. At various times the presence of cholin in the cerebrospinal fluid has been questioned, primarily on account of the methods employed. Several papers[6] have recently appeared in which the authors conclude that cholin is not present in cerebrospinal fluid or only in such small quantity that it cannot be chemically identified. From animal experiments, Handelsmann states that the symptoms produced by subdural and intracerebral injections of cholin cannot be confused with epileptic seizures; and further that cholin plays no rôle in the convulsions of this

[1] Mott and Halliburton: *Phil. Trans. Roy. Soc., B*, cxci, p. 211, 1899; cxciv, p. 437, 1901. A good review with a summary of the literature of the subject is given by Halliburton: Die Biochemie der Peripheren Nerven, *Ergebnisse der Physiologie*, iv, pps, 24–83, 1905. See also Blumenthal: Ueber Cerebrospinalflüissigkeit, *ibid*, i, pps. 285–295, 1902.

[2] Gumprecht: *Verhandl. d. Cong. f. inner. Med.*, Wiesbaden, xviii, p. 326, 1900.

[3] Donath: *Zeitschr. f. physiol. Chem.*, xxxix, p. 526, 1903; also *Deutsch. Zeitschr. f. Nervenheilk.*, xxvii, p. 11, 1904, and *Revue Neurologique*, xiv, p. 145, 1906; cf. De Buck: Pathogenie und Diagnose der Epilepsie, *Jahresber f. Tierchem.* xxxvii, pps. 669-772, 1907.

[4] Coriat: *Amer. Journ. of Insanity*, lix, p. 393, 1903; *ibid.*, lx, p. 733, 1904.

[5] Rosenheim: *Journ. of Physiol.*, xxxv p. 465, 1907.

[6] Kutscher and Rieländer: *Zeitschr. f. Geb. u. Gyn.*, xxv, p. 819, 1907. Kauffmann: *Neurol. Zentralb.*, xxvii, p. 260, 1908. Handelsmann: *Deutsch. Zeitschr. f. Nervenheilk.*, xxxv, p. 428, 1908.

disease. Kajiura,[1] relying upon Roseheim's periodide test, found cholin to be absent from the cerebrospinal fluid in cases of genuine epilepsy. It should be stated, however, that an entirely new light has recently been thrown upon the whole cholin problem by Modrakowski[2] who has found that absolutely pure cholin is physiologically inert and does not lower blood pressure, the familiar pressor effect being due to an impurity, possibly neurin. The action of this impurity in commercial cholin is antagonized by atropin, hence it is that after atropinization commercial cholin produces a rise of blood pressure, which is always the result if pure cholin is employed. Cholin is very unstable, and, as Modrakowski suggests, is apparently changed to neurin, possibly under the action of microörganisms. Pure specimens rapidly develop this impurity. From the above it is very evident that the final word has not yet been spoken on the cholin problem.

The work of Bauer[3] is of interest in this connection. Trimethylamine has been found to be a constant constituent of normal urine due to the normal degradation of lecithin substances. This author has observed that in diseases due to nervous degeneration the trimethylamine elimination is greatly augmented.

It is generally conceded that in dementia paralytica, the protein content of the cerebrospinal fluid is, as a rule, considerably increased, this increase being in a large measure due to globulins, though a trace of albumin is generally found to be present. Normally, albumin is absent. Along with the increase in protein, there is also an increase in the number, and a change in the character of the cells of the fluid.

Aside from the above, there are no definite chemical changes in the spinal fluid which have been associated with any of the various forms of insanity.

In his recent paper on cholin in the cerebrospinal fluid, Rosenheim[4] states that in all cases of acute degenerative insanity where the presence of cholin was demonstrated in the fluid, relatively large amounts of potassium could also be obtained from the alco-

[1] Kajiura: *Quart. Journ. of Exper. Physiol.*, i, p. 291, 1908.
[2] Modrakowski; *Arch. f. d. ges. Physiol.*, cxxiv, p. 601, 1908.
[3] Bauer: *Beitr. z. Chem. Physiol. u. Path.*, xi, p. 502, 1908.
[4] Rosenheim: *Loc. cit.*

holic extracts; whereas in the cases in which cholin was absent, these extracts yielded very little of the potassium platinochloride.

Considerable functional importance has recently been attached to potassium compounds in the nervous system through the microchemical researches of Macallum[1] and Macdonald.[2] Macallum, by means of his sodium cobaltinitrite reagent, found potassium to be present in the nerve fibers, but not in the nerve cells. He observed that potassium was present chiefly at the nodes of Ranvier and in the neurokeratin framework of the medullary sheath. Later Macdonald, using Macallum's reagent, found that it was not until injury of the axis cylinder took place in nerve fibers that potassium was liberated in a form which rendered it readily detectable by inorganic reagents. He concluded that since precipitates of potassium salts could be obtained at any point arbitrarily selected as the site of an injury, these salts were, therefore, really present at every point in the course of the nerve fiber, and that too in large quantity. He further observed that the presence of potassium salts was only found where the axis cylinder was involved in the process of injury. Later Macdonald and Finch[3] reported that the passage of an electrical current through a sciatic nerve led to a heavy precipitation of potassium in the nodes of Ranvier in the kathodal region, and to an absence of precipitate in, or to the presence of an external precipitate upon the nodes in the anodal region. It would seem probable that potassium was present in the nerve fibers in some organic combination. Though considerable criticism has recently been made in general of microchemical reactions, still from the above, the importance of potassium compounds in the nervous system can scarcely be disputed.

On this account it seemed of interest to note the potassium content of the cerebrospinal fluid. Comparatively few observations appear to have been made, and the table given below represents, so far as the author is aware, all work previously done on the subject. The figures have all been converted into milligrams of K_2O in 10 cc. of fluid in order to be readily comparable with subsequent data.

[1] Macallum: *Journ. of Physiol.*, xxxii, p. 125, 1905.
[2] Macdonald: *Proc. Roy. Soc.*, lxxvi, B, p. 329, 1905.
[3] Macdonald and Finch: *Journ. of Physiol.*, xxxv, p. xxxviii, 1907.

SOURCE OF CEREBROSPINAL FLUID.	MGMS. K_2O IN 10 CC. FLUID.	INVESTIGATOR.
Acute hydrocephalus	14.3	C. Schmidt [1]
" "	10.4	C. Schmidt
Hydrocephalus	8.3	Coriat[2]
Chronic hydrocephalus	6.9	Hilger[3]
" "	3.6	Salkowski[4]
Hydrocephalus	3.0	Panzer[5]
Chronic hydrocephalus	2.7	C. Schmidt[1]
Normal fluid of calf	2.4	Nawratzki[6]
Chronic hydrocephalus	2.1	Yvon[7]
Normal fluid of calf	1.9	Nawratzki[6]
Chronic hydrocephalus	1.8	Halliburton[8]
Meningocele	1.7	Zdarek[9]

The observations quoted above show wide variations which are rather difficult to explain. Though the sodium observations are not here recorded, it may be stated that the ratios between the potassium and the sodium varied with the potassium content. As early as 1850, when the first observations were made, C. Schmidt noted the difference in potassium content of the three cases he investigated, and concluded that only the cases of acute hydrocephalus were rich in potassium. It may be mentioned that the amount of potassium in Schmidt's first two cases is higher than that which occurs in blood serum and lymph. From the figures of Abderhalden ,[10] the amount of potash in the blood serum of various animals is very constant, averaging about 2.5 mgms. for 10 cc. of serum. The older analyses of C. Schmidt[11]

[1] C. Schmidt: Cited by Hopper-Seyler, *Physiologische Chemie*, Berlin, p. 604, 1881.

[2] Coriat: *Amer. Journ. of Physiol.*, x, p. 111, 1903.

[3] Hilger: cited by Salkowski.

[4] Salkowski: *Festschrift für M. Jaffé*, p. 263, 1901.

[5] Panzer; *Wiener klin. Wochenschr.*, xii, p. 805, 1899.

[6] Nawratzki: *Zeitschr. f. physiol. Chem.*, xxiii, p. 532, 1897.

[7] Yvon: cited by Salkowski.

[8] Halliburton: *Journ. of Physiol.*, x, p. 232, 1889.

[9] Zdarek: *Zeitschr. f. physiol. Chem.*, xxxv, p. 201, 1902.

[10] Abderhalden: *Zeitschr. f. physiol. Chem.*, xxv, p. 65, 1898.

[11] C. Schmidt: cited from Hammarsten's *Physiological Chemistry*, translated by Mandel, p. 239, New York, 1907.

give about 3.2 mgms. of potassium oxide for 10 cc. of human serum, with about five times that amount for the whole blood. It seems improbable that there should be such a wide variation in the potassium content of the cerebrospinal fluid under normal conditions. The cerebrospinal fluid examined by Nawratzki and by Zdarek was obtained during life, while that of Coriat was procured after death. This fact is an important one as we shall later see. Further than this, no conclusions can be drawn from the data given above.

From the results of brain analyses, Koch[1] noted that in dementia præcox there is a marked lessening of the neutral sulphur (35 per cent on the average) and a rise of inorganic sulphates, while the lipoid sulphur is not altered. He concluded from these observations that some of the symptoms of this disease could be explained by an interference with the oxidative processes. In another paper[2] he suggests that since there is in the nervous system a metabolism leading to a compound from which sulphur can be readily split off as sulphate, it would be interesting to examine the cerebrospinal fluid. From his analyses of the cerebrospinal fluid, he found sulphates to be present only in traces during life. After death, small amounts were observed, the highest figures being obtained after the lapse of the longest time post-mortem, and that too in the oldest individuals examined. The type of insanity appeared to have no influence on the quantity. Aside from the sulphate determinations in the cerebrospinal fluid reported by Koch, very few analyses have been made, though a number of isolated cases are on record, some observers obtaining only traces, others small amounts.

On account of the large amount of phosphorus in the nervous tissue, phosphorus metabolism has always been of special interest in connection with the nervous system Donath[3] states, "One sees, therefore, as I have already pointed out, a certain degree of parallelism in the occurrence of lymphocytes, protein, cholin and phosphoric acid in the cerebrospinal fluid." From his phosphate

[1] Koch: *Zeitschr. f. physiol. Chem.*, liii, p. 496, 1907.
[2] Koch: *Arch. of Neurol.*, iii, p. 331, 1907.
[3] Donath: *Journ. of Physiol.*, xxxiii, p. 211, 1905.

observations[1] on thirty different cases, he obtained average figures as follows:

Tumor cerebri	77	parts P per million[2]
Tabes dorsalis	89	" " " "
Dementia paralytica	96	" " " "

His highest individual figures were 222 parts P per million in one case of paresis. The largest amounts of protein were also found by him in tabes dorsalis and paresis. It may be noted that the cerebrospinal fluid was here removed during life for diagnostic purposes. Koch[3] also made determinations of the phosphorus, his observations showing an increase in phosphates parallel to the number of hours after death before the cerebrospinal fluid was withdrawn. Apelt and Schumm[4] likewise noted this post mortem increase in phosphates, and further concluded from their observations that there was considerable variation in the phosphorus content in health. Other investigators have estimated the phosphorus content of the fluid; but the observations cited represent perhaps the more important work on the subject.

The foregoing comprises the noteworthy chemical data on the spinal fluid with a bearing on mental diseases. The present investigation was begun at the suggestion of Prof. Lafayette B. Mendel, especially with the idea of testing the validity of Rosenheim's[5] assertion in regard to the potassium content of the fluid in dementia paralytica. Cerebrospinal fluid was obtained both during life and after death. Fluid was taken not only from the cases of paresis, but likewise for comparison from other cases which lent themselves to experiment.

In the analyses here reported, the appearance of the fluid was noted upon removal, and the volume, the specific gravity, the reaction to litmus, and the reducing power determined. In all cases the potassium was quantitatively determined, while in the latter observations quantitative determinations of the protein, phosphate, and sulphate were made.

[1] Donath: *Zeitschr. f. physiol. Chem.*, xlii, p. 141, 1904.

[2] Recalculated to my own terms for comparison.

[3] Koch: *Loc. cit.*

[4] Apelt and Schumm: Quoted from abstract in *Biochem. Centralb.*, vii, p. 757, 1908.

[5] Rosenheim: *Loc. cit.*

Where possible, the cells were enumerated, and in the cases of general paralysis an attempt was always made to isolate the diphtheroid bacillus, *B. paralyticans*, of Ford Robertson.[1]

The methods employed were as follows: the fluid was drawn with a three inch trocar possessing a mandrin, the procedure employed during life being that described by Quincke.[2] By this procedure the pressure can roughly be measured with little difficulty. As soon as possible after the removal of the fluid, its reaction to litmus was tested, and its specific gravity taken with a small hydrometer. One cubic centimeter of the fluid was employed to determine its reducing power, Benedict's[3] solution being used on account of its delicacy. When no blood was present, a cell enumeration was made, five cubic centimeters of the fluid being precipitated with twice the volume of alcohol, 96 per cent, and the precipitate being thrown down in an electric centrifuge. The volume of this precipitate was recorded as a rough protein estimation.

For the quantitative determinations, the fluid was always centrifugated, and the clear fluid used for analysis. The protein was coagulated, filtered on a Gooch crucible, dried and weighed, the sulphate next being removed as barium sulphate, and finally the phosphate determined by the molybdic method, all of which procedures are described by Koch.[4] The potassium was determined by the sodium cobaltinitrite method recently described by Drushel.[5] The method employed is briefly as follows:

If possible, two 10 cc. portions of fluid are placed in porcelain crucibles of 30 cc. capacity, 1 cc. of concentrated sulphuric acid and 4 cc. of concentrated nitric acid added, and then evaporated to as small as possible a volume over a steam bath, later being burned to a pure white ash over a

[1] Robertson and M'Rae: *Rev. of Neurol. and Psychiat.*, v, p. 455, 1907. References to preceding papers.

[2] Quincke: in *Diseases of the Nervous System*, edited by Archibald Church, translated from *Die Deutsche Klinik* by Julius L. Salinger, New York and London, 1908, p. 237. See also Ebright: *Journ. Amer. Med. Assoc.*, li, p. 1566, 1908.

[3] Benedict, S. R., This *Journal*, iii, p. 101, 1907.

[4] Koch: *loc cit.*

[5] Drushel: *Amer. Journ. of Sci.*, xxiv, p. 433, 1907; *ibid*, xxvi, p. 555, 1908, also *Zeitschr. f. anorgan. Chem.*, lvi, p. 223, 1907; *ibid*, lxi, p. 137, 1909.

Bunsen burner. After being allowed to cool, 3 to 4 cc. of warm distilled water are added to dissolve the salts and then 10 cc. of the sodium cobaltinitrite reagent and 1 to 2 cc. of glacial acetic acid. The crucibles are then allowed to stand over night in a cool place. On the following day the contents of each crucible is filtered through a newly incinerated platinum Gooch crucible with asbestos mat, washed with ice cold water, and then the mat removed and placed in a beaker containing 10–15 cc. $\frac{N}{10}$ potassium permanganate in about 100 cc. of solution nearly at the boiling point and stirred. The crucible is then immersed in the hot permanganate solution in order to oxidize the last traces of the precipitate which might have adhered to it. The crucible is now removed, washed, and the solution heated five to six minutes until manganese hydroxide begins to separate out and the solution darkens. Five to 20 cc. of 1 to 7 sulphuric acid are now added, and the solution, after stirring, allowed to stand for several minutes. A known excess of $\frac{N}{10}$ oxalic acid, containing 50 cc. of strong sulphuric acid to the liter, is next run into the beaker, after which it is titrated to color with the permanganate, the excess of permanganate over the oxalic representing the amount of potassium. Drushel employed the theoretical factor 0.000856 gms. K_2O as being the equivalent of 1 cc. strictly $\frac{N}{10}$ permanganate. After a large number of determinations with pure potassium chloride and potassium sulphate, the author decided to employ the empirical factor 0.0008017 grams K_2O as the equivalent of 1 cc. of permanganate. This, however, is much nearer the theoretical factor than the 0.000785 factor employed by Adie and Wood. The method appears to be very satisfactory. When care is taken, duplicates should always agree to a small fraction of a milligram.

The results of the investigation are summarized in the tables.

DISCUSSION OF RESULTS.

The pressures of the cerebrospinal fluid recorded in the tables show quite wide variation, three cases being considerably above the normal pressure given by Krönig[1] (120 to 180 mm.) and five much below. During life little difficulty was usually experienced in obtaining 25 cc. of fluid and no ill effects were ever observed. After death, on one occasion, over 200 cc. of fluid were removed and in several other instances large amounts were obtained. Two hundred cubic centimeters is a larger amount of fluid than the author has been able to find recorded as being drawn by lumbar puncture.

As a rule the fluid removed during life was found perfectly

[1] Krönig: cited from Rous; *Amer. Journ. Med. Sci.*, cxxxiii, p. 572, 1907.

TABLE I.

Analyses of Cerebrospinal Fluid during life.

	NAME.	DATE 1908.	AGE.	PSYCHOSIS.	PRESSURE.			AMOUNT.
					AT THE BEGINNING OF PUNCTURE.	AT THE END OF PUNCTURE.	VARIATION.	
					mm.	*mm.*	*mm.*	*cc.*
1	Mr. E. J. T.	July 28	55	Dementia paralytica.	385	120	265	25
2	Mr. D. D.	Aug. 5	48	"	180			5
	"	Sept. 20			65	10	55	18.5
3	Mr. A. M.	Aug. 5	37	"	150	40	110	26.5
4	Mr. G. F. G.	Aug, 7	55	"	180	70	110	26
5	Mr. W. F. H.	Aug. 7	44	"	110	60	50	25
6	Mrs. E. C.	Aug. 10	54	"	75	20	55	27
	"	Sept. 9			120	30	90	17.5
7	Mr. L. W.	Aug. 11	39	"	60	00	60	16
8	Mr. W. B.	Sept. 20	26		120	40	80	40
9	Mr. S. R. L.	Sept. 20	50	"	230	110	120	16
10	Mr. W. N.	Oct. 17	32	"	240	70	170	28
11	Mr. J. P. E.	Oct. 17	60		90	45	45	31
12	Mr. B. D. K.	Aug. 11	36	Epileptic insanity	145	100	45	35
13	Mr. W. C.	Aug. 11	32	"	160	80	80	24
14	Mrs. M. L.	Oct. 13	33	Alcoholic pseudoparesis	130	60	70	32
15	Mrs. I. H.	Oct. 13	49	Korssakow's psychosis	70	40	30	17

TABLE I.

Analyses of Cerebrospinal Fluid during life.

APPEARANCE.	SPECIFIC GRAVITY.	REACTION TO LITMUS.	REDUCTION WITH BENEDICT'S SOLUTION.	VOLUME OF ALCOHOL PRECIPITATE.	PROTEIN PER CENT.	POTASSIUM MGMS. K_2O IN 10 CC.	P PARTS PER MILLION.	S PARTS PER MILLION.
				p. ct.				
Perfectly clear		Faintly alkaline	Moderate	2		2.2		
Tinge of blood		"	Slight			2.8		
Perfectly clear	1.006	Very faintly alkaline		3	0.186	3.4	61	Trace
Tinge of blood		Faintly alkaline	Slight			2.1		
Perfectly clear	1.007	"	"	2		1.8		
Perfectly clear	1.007	"	Very slight	1.6		2.0		
Very slightly turbid	1.006	Very faintly alkaline	Moderate	1		1.8		
Perfectly clear	1.006	Faintly alkaline		2	0.062	2.2	70	Trace
Slightly clouded	1.006	"	Considerable	1.6		1.7		
Perfectly clear	1.008	Very faintly alkaline	Slight	2	0.061	3.3	22	Trace
Trace of blood	1.006	"	Strong		0.098	3.0	78	Trace
Trace of blood	1.006	Faintly alkaline	Moderate		0.011	2.3	26	Trace
Perfectly clear	1.006	Faintly alkaline	Moderate	4	0.143	2.1	25	Trace
Perfectly clear	1.005	Very faintly alkaline	Much	0.7		1.7		
Slightly clouded	1.006	"	Trace	0.7		2.2		
Perfectly clear	1.006	Faintly alkaline	Moderate		0.013	2.1	31	Trace
Trace of blood	1.006	"	Slight		0.005	2.5	11	8

TABLE II.

Analyses of Cerebrospinal Fluid after Death.

	NAME.	DATE 1908.	AGE.	PSYCHOSIS.	CAUSE OF DEATH.	HOURS POST MORTEM.	AMOUNT CC.
1	Mr. E. W.	June 25	46	Dementia paralytica	Dementia paralytica	6½	130
2	Mr. T. McG.	Sept. 25	43	"	" "	21	22
3	Mr. A. G.	Oct. 9	47	"	" "	½	192
4	Mr. T. W.	Oct. 10	37	"	" "	6½	94
5	Mr. A. M.	Oct. 13	37	"	" "	4	177
6	Mrs. J. D.	June 29	45	Epilepsy	Epileptic convulsions	9	42
7	Mr. M. B.	July 8	41	Alcoholic delusional insanity	Entero-colitis	15	106
8	Mr. T. B.	Sept. 28	59	"	Bronchopneumonia	8	99
9	Mr. J. M.	July 9	77	Senile dementia	Valv. disease of the heart	1	107
10	Mrs. L. D.	July 14	74	"	Entero-colitis	8	75
11	Mrs. B. H.	Sept. 27	74	"	Arteriosclerosis	4½	112
12	Mr. T. B.	Oct. 8	78	"	Valv. disease of the heart	15	25
13	Mrs. S. J.	Sept. 12	48	Organic dementia	Brain tumor	14	19
14	Mrs. M. L.	Sept. 26	61	Melancholia	Exhaustion	12	25
15	Mrs. M. S.	Sept. 29	42	Manic-depressive Dep. Phase	Mitral regurgitation Chronic nephritis, phthisis	11	42
16	Mr. E. P.	Oct. 8	71	Dementia præcox, Hebephrenic Form	Valvular disease of the heart	3	202
17	Mrs. A. T.	Oct. 9	65	"	" " "	7	37

TABLE II.

Analyses of Cerebrospinal Fluid after Death.

APPEARANCE.	SPECIFIC GRAVITY.	REACTION TO LITMUS.	REDUCTION WITH BENEDICT'S SOLUTION.	VOLUME PER CENT OF ALCOHOL PRECIPITATE.	PROTEIN PER CENT.	POTASSIUM MGMS. K_2O IN 10 CC.	P PARTS PER MILLION.	S PARTS PER MILLION.
White, slightly turbid		Slightly alkaline	Strong	Much		8.4		
Slightly turbid	1.009	Faintly alkaline	Slight		0.095	11.9	150	Trace
Very slightly turbid	1.008	Very faintly alkaline	Considerable	1.8	0.061	12.0	83	Very faint trace
Slightly turbid (cells)	1.008	Faintly alkaline	Moderate	1.8	0.048	9.7	74	Trace (.5)
Turbid (cells)	1.009	Faintly alkaline	None	2.	0.079	8.3	74	Faint trace
Clear	1.009	Slightly acid	Trace	Small amount		7.0		
A portion bloody	1.008	Faintly alkaline	None	Considerable		12.7		
Very slightly turbid	1.008	Faintly alkaline	None		0.030	10.3	34	Trace
Trace of blood		Faintly acid				9.9		
A portion bloody	1.009	Faintly acid	Moderate			10.6		
Slightly clouded	1.008	Neutral	Strong	2.	0.114	12.2	192	Trace
Slightly yellow	1.007	Neutral	None		0.055	8.7	91	Trace
Turbid very slightly yellow	1.007	Faintly acid	Very slight		0.286	7.2	86	34
Clear	1.008	Very slightly alkaline	None	2.5	0.148	10.7	120	Trace
Slightly clouded	1.008	Very slightly acid	None	1.5	0.021	10.7	89	Trace
Very slightly turbid	1.007	Faintly alkaline	Slight	1.	0.043	7.0	74	Small amount
Slightly yellow	1.007	Faintly alkaline	None		0.325	9.7	85	Trace

clear, though in two instances it appeared to be slightly clouded with cells (lymphocytes). After death, the fluid was more often observed turbid than not. During life, the specific gravity was found more usually to be 1.006, but the post mortem increase in inorganic salts appeared to raise this figure to about 1.008. The reaction of the fluid was always found to be faintly alkaline to litmus during life and generally so after death, though this was not always the case.

The fluid was found to give at least a trace of reduction with Benedict's solution in all cases during life, though in the tests made post mortem this was not always observed. Since the reducing body of the cerebrospinal fluid is now generally regarded as dextrose and not pyrocatechin, as originally stated by Halliburton,[1] no further study of this point was made.

From the few data above reported, the protein content of the fluid during life was found much higher in paresis than in the other types of insanity examined. The fluid withdrawn after death showed wide variations and no conclusions can be drawn, though the case of brain tumor appears noteworthy. In the analyses of fluid removed during life, the protein content varied from 0.005 to 0.186 per cent, with an average of 0.072 per cent for eight observations, and, after death from 0.021 to 0.325 per cent, with an average of 0.109 per cent for twelve observations. Apparently the protein of the fluid increases after death along with the other constituents of the fluid. There are a large number of observations on the protein content of the fluid in the literature, but so many different methods have been employed that the results are hardly comparable. However, the figures recorded above are similar to those obtained by Koch.[2]

The phosphate determinations cannot be correlated with any of the various types of insanity. A marked increase was noted after death, as was the case in the observations of Koch. Koch's observations showed an increase in phosphate parallel to the number of hours after death at which the fluid was drawn. This was not quite so noticeable in my own observations.

In the sulphate determinations it was impossible to obtain

[1] Halliburton: *Journ. of Physiol.*, x, p. 232, 1889.
[2] Koch: *Loc cit.*

more than a trace of precipitate, though this always appeared characteristic. In general, there seemed to be a larger quantity present after death, but it was impossible to detect more than a trace even in fifty cubic centimeters of fluid. There were two exceptions to this general statement. In the case of brain tumor, a comparatively large quantity was obtained (34 parts per million), and in the case of Korssakow's psychosis during life (8 parts per million).

Aside from the potassium observations reported in the general table, several other less complete examinations were made. These are reported in the table below. All are postmortem observations.

NAME.	AGE.	PSYCHOSIS.	CAUSE OF DEATH.	HOURS POST-MORTEM.	VOL. CC.	REACTION TO LITMUS.	K_2O MGMS. IN 10 CC.
Mr. G. R...	40	Dementia paralytica	Dementia paralytica	14	85.	Slightly alkaline	11.9
Mr. L. W...	39	"	"	8	4.8	"	6.9
Mr. F. M...	78	Senile dementia	Valvular disease heart	18	20.	"	7.8
Mr. G. W..	58	Imbecility	"	36	13.	Neutral	9.3
Mr. D. La J.	60	Dementia præcox	"	4	125.	"	8.6

In the potassium estimations which are here recorded, one fact is very prominent. After death, the quantity of the potassium in the cerebrospinal fluid is greatly increased. The average of 17 analyses during life was 2.3 mgms. per 10 cc. of fluid, while the average of 22 analyses after death was 9.7 mgms. per 10 cc., an increase of over four times after death. No such increase in the potassium content of the fluid was observed in the examination of 15 living individuals suffering from dementia paralytica. In the 7 cases of paresis which it was possible to investigate after death, the same postmortem increase was noted as found in all other cases observed after death. In two cases of dementia paralytica, it was possible to examine the fluid both during life and after death, viz: Mr. A. M., and Mr. L. W. The first case increased from 2.1 mgms. during life to 8.3 mgms. after death, while in the

second case there was an increase from 1.7 mgms. to 6.9 mgms. The various cases examined represented all stages of the disease. In light of the above data, the increase in potassium which Rosenheim[1] noted will probably find explanation in postmortem change rather than in the degenerative changes which are found in dementia paralytica. At any rate, according to the observations recorded, the potassium is greatly increased after death, and that too at once, since as high figures were obtained a half hour postmortem as at any time later.

This increase in potassium may find explanation in the fact that when a cell dies certain physical changes occur which are probably of considerable importance. The permeability of the cell wall is almost immediately increased, so that all diffiusible substances readily pass through it and its semipermeable character is lost. Macdonald and Finch[2] have shown that nerves which are placed in saline containing chloroform tend to have the potassium salts limited in their distribution. Macdonald[3] has further shown that injury and death of nerves liberate electrolytes. Roaf and Anderson[4] found that when emulsions of sheep's brains were treated with ether, chloroform, carbon dioxide or $\frac{M}{20}$ acetic acid, the electrolytic conductivity was increased. In other words, dead or dying portions of nerves have the electrolytes liberated from their "masked" condition. The potassium may be held either in some organic combination or inside cell walls, possibly in the nervous system. Assuming potassium to be one of the constituents of the nervous tissue, it is apparently speedily liberated from this condition after death. It should further be borne in mind that the potassium content of the cerebrospinal fluid during life corresponds very closely to the amount of potassium in the blood serum, while after death the quantity of potassium in the cerebrospinal fluid agrees more nearly with that of the whole blood.

[1] Rosenheim: *Loc. cit.*
[2] Macdonald and Finch: *Loc. cit.*
[3] Macdonald: *Loc. cit.*
[4] Roaf and Anderson: *Biochem. Journ.*, ii, p. 412, 1907.

SUMMARY AND CONCLUSIONS.

The cerebrospinal fluid of 15 insane patients was examined during life for diagnostic purposes, and 22 observations were made on fluid removed after death.

The data reported all indicate that changes take place rapidly in the cerebrospinal fluid after death, and consequently results obtained with postmortem fluid cannot be compared with fluid obtained during life.

The fluid was found to give at least a trace of reduction with alkaline copper solutions in all cases during life, though in the tests made postmortem this was not always observed.

During life the protein content of the cerebrospinal fluid was observed to be increased in dementia paralytica. Along with the postmortem rise in inorganic constituents, there was also noted an increase in the amount of protein.

Much higher values were obtained for phosphates inthe fluid removed after death than in the fluid drawn during life.

Sulphates were never found to be present in more than traces either during life or postmortem.

Little variation was noted in the potassium estimations made during life, but after death the potassium content of the fluid was always increased at once, and that about four times. A new method of estimation was employed.

The marked postmortem rise in inorganic constituents leads, as would be expected, to an increased specific gravity.

HUMAN PANCREATIC JUICE.

By HAROLD C. BRADLEY.

(From the Department of Physiology, University of Wisconsin.)

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The opportunity for the examination of human pancreatic juice occurs so rarely, and the amounts of material available for chemical analyses are usually so small, that any additional material may be of considerable interest in determining the specific characters of the human secretion. Such an opportunity has recently been presented to this laboratory through the kindness of Dr. Reginald Jackson of Madison, to whom we wish here to express our thanks.

Mr. B., a farmer of 45 years, was severely kicked by a horse in the abdomen, just over the spleen. He apparently recovered from the blow immediately and after a day or two experienced little or no pain. A swelling was noticed later which increased steadily and exerted considerable pressure upon the stomach. Nausea and vomiting resulted, and the patient was finally unable to retain food. He presented himself to Dr. Jackson, some weeks after the accident, greatly emaciated and weak. A median incision developed a large cyst as the result of rupture of one of the pancreatic ducts and over a liter of fluid was removed. Drainage was kept up for about four weeks, the flow gradually decreasing and finally stopping. After the operation the nausea disappeared and the patient regained his appetite. His food consisted of three meals a day, of regular ward diet containing meat, fats, etc. Digestion and utilization were apparently unimpaired and Mr. B. rapidly regained his normal weight, color and strength. At the end of six weeks he left the Madison Hospital and returned to work on his farm.

A preliminary examination showed that the juice was alkaline to litmus, powerfully amylolytic, and contained a considerable amount of coagulable protein. The early samples were quite inactive toward fibrin or gelatin but could be activated by the addition of a few drops of an intestinal extract prepared from the duodenal mucosa of a dog. The juice was clearly of pancreatic origin and after the first few days was probably quite normal.

This assumption is based on the condition of the patient following the operation. All sorts of food were taken and digested with perfect ease, including the fats which were always present in the mixed ward diet. Since digestion and utilization were quite normal we may assume that the pancreatic secretion, a portion of which we were able to collect outside, was normal. As further confirmation of this assumption, the following pages will demonstrate that in many points it resembles human pancreatic juices reported elsewhere as normal, and also that of dogs.

An attempt was made to measure rate of flow from period to period, but in many instances leakage into the dressings and losses for other reasons render the figures of little significance. It may also be questioned whether the rate of flow from a ruptured side duct would necessarily parallel that from the entire gland. Certain samples were especially collected however to determine whether the flow before and after meals was appreciably different. From samples III and IV it is evident that the rate of flow is increased after the taking of a meal, just as has been observed often by others.

The specific gravity was measured by spindle at 20°. The figures show great constancy and are all slightly higher than those observed by Glaessner[1] which average about 1007.

Alkalinity. The matter of alkalinity seems to have been passed over somewhat hastily by investigators. Herter[2] states that the human juice obtained by him was strongly alkaline. Kudrewetzky[3] found the alkalinity of dog's pancreatic juice to vary from 0.05 to 0.89 grams of hydrochloric acid per 100 cc.;—that is, from $\frac{N}{70}$ to $\frac{N}{5}$. Glaessner records an alkalinity toward phenolphthalein of from 1 cc. to 3.5 cc. $\frac{N}{10}$, in 10 cc. of the human juice. This represents the alkalinity due to normal carbonates only and is considerably higher than our own findings. He does not however determine whether there is alkalinity due to salts like bicarbonates. Foa[4] has shown that the alkalinity measured by concentration of hydroxyl ions, using electrometric methods, was about $\frac{N}{10000}$ potassium hydroxide for the pancreatic juice of

[1] *Zeitschr. f. physiol. Chem.*, xl, p. 465.
[2] *Ibid.*, iv, p. 160.
[3] Cited from Tigerstedt's *Text Book of Physiology*.
[4] *Compt. rend. soc. biol.*, lviii, p. 867.

dogs. In general the data available upon this subject are incomplete and scanty so that it was considered worth while to examine this juice a little more closely. Electrometric methods were not available so that titration with various indicators was used instead. Many samples were found to be alkaline to phenolphalein, a few were not. All were alkaline to litmus, methyl orange and hæmatoxylin. All grew more distinctly alkaline to phenolphthalein upon boiling, and the discharge of the pink color by adding acid to a boiling solution was only temporary. It was assumed that the greater part of the alkalinity was therefore due to carbonates and bicarbonates, and differential titrations were made. Phenolphthalein was used to indicate the amount of normal carbonate present; methyl orange indicated the bicarbonate content (a small fraction of which comes from the original carbonate of course, in this case too small to be of any moment). The figures tabulated below in Table I represent the amount of $\frac{N}{10}$ acid required to neutralize the alkalinity of 10 cc. of the juice. Many samples were checked by titrating hot with hæmatoxylin, by continued boiling and titrating with acid till no more alkalinity to phenolphthalein developed, and by noting in these solutions the

TABLE I.

NO.	CHARACTER.	SP. GR.	VOL.	$\frac{N}{10}$ Na_2CO_3	$\frac{N}{10}$ $NaHCO_3$ cc.
I.....	Cystic fluid	1010	700+	0.00	3.10
II.....	24 hour	1011	650	0.40	10.40
III.....	2 hour	1012	52	0.90	10.40 before meal
IV.....	4 hour	1010	155	0.00	9.5 after meal
V.....	20 hour	1009	635	0.45	9.8
VI.....	4 hour	1010	167	0.55	8.6 after meal
VII.....	20 hour	1010	420	0.45	7.45
VIII.....	20 hour	1010	545	0.10	6.80
IX.....	4 hour	1011	88	0.30	6.50 after meal
X.....	24 hour			0.10	5.60
XI.....	24 hour			0.00	5.80
XII.....	24 hour			0.45	5.75
XIII.... ..	24 hour			0.25	5.90
XIV.....	24 hour			0.40	7.00
XV.....	24 hour			0.55	6.10
XVI.....	24 hour			0.30	6.85
XVII.....	24 hour			0.15	7.05

point at which the proteins coagulate out. The latter is of course but a rough indication of the approach to neutrality, the other two more exact methods for determining the total alkalinity agreed well with the tabulated figures. It was evident that the alkalinity of the juice was produced by carbonates and bicarbonates almost entirely; the amount of alkaline phosphates present must have been of small significance. It is of importance to note how small is the proportion of normal carbonate to the total alkalinity, or bicarbonate.

THE ENZYMES.

Rennin, *invertase*, and *lactase* were not found in any of the samples obtained. Lactase was tested for by allowing the juice to stand in contact with a lactose solution for several hours under toluol. At the end of that time no dextrosazone could be detected. In the same way invertase was tested for by means of cane sugar followed by a Fehling's determination of reducing sugar. The juice would not curdle milk. These results are in accord with those of Glaessner and seem to still further discredit the notion of pancreatic adaptability, since neither invertase nor lactase could be found although the patient was accustomed to sugar and to large amounts of milk in his diet.

Amylopsin was abundant and seemed to be fairly constant in amount from day to day. A few experiments only were tried quantitatively since this enzyme seemed to be the best known and perhaps least interesting of the pancreatic ferments. Those results which were obtained are tabulated below.

Ten cubic centimeters of a 1 per cent starch paste were taken and 5 cc of a juice diluted 1:10 added. The digestions were allowed to run at 40° until duplicates showed no further blue with iodine solution. All the small flasks were then plunged together into boiling water to arrest digestions at the same moment. They were then made up to 50 cc. and run in from a burette into a boiling Fehling's titration solution. The reduction of copper was indicated on the outside by a mixture of potassium iodide, starch paste, and acetic acid. The number of cc. required to reduce the copper will then contain 0.05 gram of dextrose or its equivalent of maltose. Where the amount of sugar in the digestion was less than that required to reduce the copper a standard dextrose solution could be added, 10.7 cc. of which contained 0.05 gram. of dextrose.

TABLE II.

JUICE.	STARCH.	TITRATION.	SUGAR.
	cc.	*cc.*	*per cent.*
I	10	36.5	0.13
II	10	36.0	0.13
III	10	35.6	0.14
IV	10	36.0	0.13
V	10	37.5	0.13
VI	10	35.0	0.14
Water	10	50.0 + 10.7 stand.	0.00

Another series of digestions was made to discover what effects bile salts and manganese sulphate might have. Gignon and Rosenberg[1] have recently reported a marked acceleration of pancreatic amylopsin in the presence of salts of iron and manganese, and bile has been found to accelerate so many enzymic processes that it was interesting to see whether it would also influence pancreatic amylopsin. Table III contains the results of these experiments.

TABLE III.

JUICE II.	5 CC. SOL. ADDED.	TITRATION.	SUGAR.
cc.		*cc.*	*per cent.*
10	Bile salts 10 per cent	20.60	0.242
10	" " 5 "	20.00	0.250
10	" " 2.5 "	23.40	0.213
10	" " 1.25 "	20.80	0.244
10	" " 0.63 "	20.10	0.25
10	" " 0.31 "	18.30	0.277
10	$MnSO_4$ $\frac{N}{10}$	25.40	0.196
10	$MnSO_4$ $\frac{N}{20}$	20.00	0.25
10	$MnSO_4$ $\frac{N}{50}$	20.65	0.25
10	$MnSO_4$ $\frac{N}{100}$	21.50	0.24
10	$MnSO_4$ $\frac{N}{1000}$	18.90	0.26
10	Water	18.30	0.277

Instead of acceleration by manganese and bile salts we have found some slight inhibition. It must be pointed out however that the experiments cited are open to one pertinent criticism.

Loc. cit.

The digestions were carried too far to show differences of speed in the initial stages of the reactions. Unless a substance affects the final equilibrium of a digestion no clue as to its temporary effect can be had from an examination of the digestion which has already approached equilibrium. It is certain too that conditions which affect final equilibrium in one direction may have the opposite effect in the earlier stages of the reaction. It is reasonable to suppose that the converse is also true, that conditions may alter initial speeds considerably without seriously changing the level of final equilibrium. It is entirely possible therefore that bile salts and those of manganese may accelerate the digestion of starch to begin with without altering equilibrium. Our results show at least that in these digestions with human pancreatic juice manganese salts exert a depressing effect upon the equilibrium point; bile salts do the same but to a lesser extent.

Trypsin and trypsinogen. Much uncertainty still prevails in regard to the status of trypsin and its precursor. Can trypsinogen be activated by substances other than enterokinase? Does normal pancreatic juice ever contain trypsin? Is there any indisputable evidence of pancreatic adaptation to diet? These are questions which may still be considered open despite the eminence and positiveness of the authority which has attempted their settlement. As to the first question, our own results answer it directly in the affirmative, for we have frequently found our samples to be strongly active proteolytically, and yet the chance of intestinal contamination in this case was out of the question. We should have to suppose a regurgitation of intestinal juice far up the main pancreatic duct and into the ruptured side duct before this activation could occur. The fact that the ruptured duct was practically always flowing—though very slowly at some times of course—makes such a regurgitation very difficult to imagine. The fact remains however that about half of the samples collected would digest gelatin and fibrin without further activation. Apparently then something besides intestinal juice has activated the trysinogen.

Furthermore several samples which were at first quite inactive developed activity upon long standing. Juice I for example showed not the slightest digestive power for a week or more. After some three weeks had elapsed it was found to be highly

active. This may have been due to bacteria, though the juice was collected under surgical conditions, into a bottle sterilized with boiling water and washed out with alcoholic thymol before the collection. The juice itself was saturated with both chloroform and toluol, excess of the two reagents remaining at the bottom and on top of the sample. Obviously while we cannot say positively that bacteria were not responsible for this activation it seems scarcely reasonable to attribute it to organisms growing in so uncongenial an environment. According to Glaesner[1] bacteria can activate trypsinogen; so also can Witte's peptone to judge from his own tables. The activation was hardly comparable to that produced by enterokinase however,—though the fact is of itself interesting since it shows the partial activation of the juice by something other than enterokinase. Karl Mays[2] found in working upon dog's pancreatic juice that standing at room temperature or above served to render inactive samples quite active. This result he frequently obtained overnight at the higher temperature. The slowness of our own activations may have been due to the fact that they were kept close to 0°. Many other investigators have reported activation from one method or another, but since we have not been able to attempt their verification they should not perhaps enter into the discussion of facts developed by our examination of human juice. It seems to us probable however that a number of substances entirely different in origin from enterokinase may produce an activation of trypsinogen. Furthermore such activation may go on normally at times in the gland itself, so that the juice as it flows from the duct may contain trypsin. As was said before, in this case about one-half of the samples were active to begin with.

All the samples collected were capable of activation by the intestinal extracts of dogs and cats. There was no evidence of a specific enterokinase necessary for the activation of human trypsinogen. This result was in every instance so sharp and positive that we are at a loss to account for the opposite results observed by Glaessner on any other basis than that the dog whose intestinal mucosa he extracted was temporarily devoid of enterokinase.

[1] *Loc. cit.*

[2] *Zeitschr. f. physiol. Chem.*, xlix, p. 188.

He found that the human juice could be activated only by an enterokinase preparation from a human intestine—a result which pointed directly to the idea of specific enterokinases. We have used all of our inactive samples and have been able to show immediate activation toward gelatin and fibrin by the addition of a few drops of intestinal extracts prepared from four dogs and one cat. In not one case has there been failure to activate.

As to the effect of diet upon the composition of pancreatic secretion we have little to offer. Nearly all our collections were for twenty-four hour periods, and they show a wide variation in amount, alkalinity, and enzyme content. Two samples only represent the juice just before a meal and just after. No. III was collected for two hours preceding a meal. The flow was sluggish, averaging 26 cc. per hour. Juice No. IV was collected during the next four hours. It averaged nearly 40 cc. per hour. A glance at table I again will show that as the juice flows slowly it also flows in concentrated form. It contains the highest amount of carbonate and bicarbonate, its specific gravity is maximum, and as will be shown in a later table it contains practically the maximum content of lipase. No. IV is much more dilute; its carbonates are absent and its bicarbonates low; its lipase is more dilute than in III. So far as our evidence goes then it is in support of Wohlgemuth[1] who could find no other adaptation to diet than that a rapid secretion was usually a dilute secretion, both in solids and enzymes, and that the diets therefore which called out the most rapid secretion produced a corresponding dilution of the enzymes. This result is quite contrary to the reported findings of the Pawlow school, Glaessner, and others who believe in a direct relation between diet and enzymes necessary to digest that diet.

In the following table (IV) is presented the comparative proteolytic power of the several samples. Series I was made directly after the collection of No. XI, though each juice was tested at once for proteolytic activity with a bit of fibrin. Series II was made after drainage had ceased, about two weeks after series I. In order that the two series might be compared No. I was used as the control in each.

[1] *Berlin. klin. Wochenschr.*, xliv, p. 47.

In making the digestions, 10 cc. of the sample was placed in a small bottle. The bottles were closed with perforated corks through which the gelatin tubes could be thrust. The digestions were kept at room temperature under toluol. A 48-hour period was allowed for determining the trypsin; then 1 cc. of an enterokinase solution was added to each and a second 48-hour period conducted. The difference in the rate of digestion —measured in mm. of gelatin dissolved—was then used for estimating the relative amounts of trypsin and trypsinogen.

TABLE IV.

Series I.

NO.	TRYPSIN.	TOTAL.	TRYPSINOGEN.	REMARKS.
I	0	11.5	11.5	Became gradually active on standing
II	0	9.	9.	" " " "
III	.	.	.	
IV	0	6.	6.	" " " "
V	2.	4.	0*	
VI	0.5	7.	6.	
VII	1.0	3.	1.	
VIII	7.	11.5	0–	
IX	7.	12.	0–	
X	1.5	3.	0	
XI	0	8.	8.	" " " "

Series II.

I	5.5	10.5	0	Self activated
XII	0	6.	6.	Became active on standing
XIII	3.5	6.5	0	
XIV	0	5.	5.	" " "
XV	0	4.	4.	" " "
XVI	0	5.	5.	" " "
XVII	0	5.5	5.5	" " "

* Since the trypsin is represented by a two days digestion twice this amount must be subtracted from the total four day digestion to indicate the trypsinogen. In some digestions the activity is slightly less during the second two day period, as though the trypsin were itself being destroyed. Thus in Series II, No. I, not only has the trypsinogen disappeared but the resultant trypsin is half gone.

Lipase. Our knowledge of the action of lipases upon esters has been enormously increased during the last few years. So much indeed has been done that any *résumé* of the multitude of isolated facts would be out of place here. A review of the facts

relating to acceleration and inhibition does not afford us yet any very clear insight into the actual mechanism of the reaction. Certain substances accelerate, others inhibit, still others exhibit both properties when the dilutions are properly chosen. Some of this work we have checked with our samples of human secretion, and in certain directions have pushed it further to collect new data upon the action of lipase.

The relative lipolytic activity of the juices was determined after drainage had ceased. This perhaps should not be interpreted to mean that the original secretion just as it flowed had this same degree of activity, though our observations have shown that the lipase in these samples does not show any appreciable alteration after many weeks of standing. Olive oil and ethyl butyrate were used giving a double series of observations. If the digestion of these two esters is accomplished by the same enzyme, we should expect the two series to closely parallel each other—if the other factors are alike. Within the limits of the experiments where end points are not sharp and emulsification is important, the two series do show remarkable similarity.

In these and the following experiments the method was essentially the same. The description here will therefore suffice for the rest. Small glass stoppered bottles were used to contain the digestions where the volume of fluid was not over 30 cc. The lipase solution was measured in and diluted with a measured amount of water or solution of bile or salts, etc. In some cases the original juice was diluted to begin with 1:10. The zymolite was added finally, the bottles shaken alike and set aside in the thermostat at 40°. Where ethyl butyrate was the zymolite a $\frac{N}{20}$ solution of sodium hydroxide in water, was used to titrate the acidity developed. Where olive oil was digested an alcoholic potassium hydroxide solution was used and alcohol (neutral) added to the titration to facilitate the reaction. Phenolphthalein was used as the indicator throughout. The figures in the subsequent tables are centimeters of $\frac{N}{20}$ acid liberated in the digestions, except where otherwise designated.

As will be seen from an examination of Table V every decided change in activity as exhibited by the digestion of olive oil is accompanied by a similar change in activity manifested toward ethyl butyrate. The agreement is not exact nor should we expect it to be since we have no assurance that the emulsion produced by a given number of shakes in one sample will be exactly like that produced in another. Indeed since we know

TABLE V.

One cc. juice + 10 cc. water + 20 drops ester in each.

NO.	DIGESTION OF OLIVE OIL.	DIGESTION OF ETHYL BUTYRATE.
I	5.10	0.50
II	12.00	2.90
III	12.80	2.40
IV	11.15	2.30
V	0.75	0.15
VI	8.70	2.75
VII	6.50	1.05
VIII	2.55	0.36
IX	1.40	0.10
X	3.10	0.10
XI	2.90	0.10
XII	11.30	1.80
XIII	2.90	0.55
XIV	10.90	1.10
XV	10.00	1.15
XVI	9.00	1.50
XVII	9.70	2.50

that there are differences in solid content and in carbonates we may be quite sure that the emulsions cannot be exactly alike. They were evidently nearly alike since the two digestion series approximately parallel each other.

While it is perhaps self-evident that the degree of emulsification will be a determining factor in reactions of heterogeneous mixtures such as these, it may not be out of place to introduce figures to show what may be the magnitude of differences produced by this variable. Table VI shows the effects of various typical emulsifying agents upon a series of digestions.

It will be noted that with moderate shaking bile facilitates the reaction when it is moderately dilute. A dilute bile solution is not superior to water itself. Gum arabic also facilitates digestion by its emulsifying ability, though it exerts at the same time an inhibitory action which is masked except where shaking is frequent. Under those conditions the advantage of the digestion containing an emulsifying agent is largely diminished, and under those conditions water is apparently as good a medium as bile for

TABLE VI.

10 cc. juice II diluted 1:10.

NO.	5 CC. SOLUTION.	$\frac{N}{20}$ ACID LIBERATED.	REMARKS.
1.....	5 per cent bile	10.70	20 shakes
2.....	1 " "	7.85	20 "
3.....	water	7.85	20 "
4.....	10 per cent gum arabic	8.60	20 "
5.....	2 " " "	8.30	20 "
6.....	0.1 per cent Ivory soap	0.00	20 "
7.....	0.02 " " "	6.50	20 "
8.....	water	7.50	2 "
9.....	5 per cent bile	11.30	2 "
10.....	water	15.40	frequent shaking
11.....	5 per cent bile	14.80	" "
12.....	10 per cent gum arabic	10.50	" "

digestion; better than a gum arabic solution. This is a very good illustration of the fact that under one set of conditions a substance may be an accelerator, while under another it may inhibit digestion. The effect of the soap solution was unexpected, and we have not attempted to discover what substance the marked inhibition was due to. It may have been the free sodium hydroxide which is sometimes present in small amounts and a subsequent table will show the effects of a small amount of hydroxide on this reaction.

From the figures above bile does not seem to play any very important rôle in the digestion of the fats, provided emulsification is good. In the digestions of other esters bile salts have been found essential to the digestion, and there the action of the bile can hardly be a physical one only. Whether it acts merely in its capacity of an excellent emulsifying agent in the digestion of olive oil or whether it has also a chemical effect may be determined by noting the nature of the variations produced by a changing concentration of the bile. If the effect is purely physical a steady change in the bile present should produce a correspondingly regular variation in digestion. If a chemical factor is involved it frequently will be disclosed by some sudden change in the degree of digestion as a definite concentration is reached and passed. If plotted, the curve of digestion should show sudden

changes at this point of concentration. A number of digestion series were therefore carried out for the purpose of detecting this chemical influence of bile on the digestion of olive oil. Whole bile was used and assumed to contain 10 per cent of bile salts; purified bile salts free from lecithin kindly furnished me by Dr. Loevenhart, were also used (I and II). No difference in their effect could be detected.

TABLE VII.

Olive Oil.

NO.	5 CC. BILE SALT SOLUTION.	I.	II.	III.
1	10. per cent.	15.60		
2	7.5 "			13.45
3	5. "	15.50	15.70	12.00
4	2.5 "		13.75	11.70
5	1.25 "		12.50	
6	1. "	12.15		9.00
7	0.5 "	10.80	10.45	8.65
8	0.3 "		11.55	
9	0.1 "	10.45	11.15	8.70
10	0.05 "	9.10		7.75
11	0.04 "		10.25	
12	0.02 "		10.55	
13	0.01 "	11.05	10.70	8.50
14	0.005 "			8.40
15	0.001 "			9.55
16	water	10.85		10.15

From the three typical digestions tabulated it is apparent that an increase in the amount of bile produces a corresponding increase of digestion. In very dilute solutions there is a slight indication of the opposite effect though very little stress should be laid upon this point. It is of more significance however when compared with the digestion of ethyl butyrate where at approximately the same dilution we have a marked sudden change in the curve. In all three the minimal digestion occurs when 5 cc. of 0.05 per cent bile salt solution is added. Change in concentration in either direction seems to produce an increased digestion. The effect of bile salts in a detailed examination of the progress of digestion will be reported in a later table. It will

be seen there that the bile exerts its effect not so much in increasing the initial speed of digestion but in raising the level of the final equilibrium between fatty acid liberated and neutral fat remaining. This effect may possibly be due to increased mass action through more perfect emulsification, or it may also be due in part to a protecting effect exercised by the bile salts upon the enzyme, so that the inhibitory action of the oleic acid is delayed.

The effect of bile upon the digestion of ethyl butyrate was found to present some striking features. The bile salts undoubtedly facilitate the emulsification of the ester just as they do triolein, but this effect is entirely lost at certain concentrations because of a much more pronounced chemical factor. This sudden and decided change is shown in the following digestions—a few from quite a number made to test this point.

TABLE VIII.

Ethyl Butyrate.

NO.	5 CC. BILE SOLUTION.	I.	II.	III.	IV.	V.
1.....	40. per cent.	23.50				
2.....	20. "	20.40				
3.....	10. "	15.20	15.76	11.05	16.80	
4.....	7.5 "	.	13.45	10.00	15.10	
5.....	5. "	15.20	9.95	8.50	11.45	
6.....	2.5 "	.	8.40	6.60	9.55	
7.....	1.0 "	11.25	7.45	6.00	8.10	5.85
8.....	0.5 "		10.20	7.95	12.35	8.25
9.....	0.05 "		16.75	9.00	22.35	8.85
10.....	0.005 "		7.70	8.45	13.85	4.45
11.....	0.0005 "		2.65	1.75	13.30	4.20
12.....	water		5.05	4.85	13.85	5.75

It will be seen that from 40 per cent of bile salts down to 1 per cent the acid liberated varies regularly in the different digestions. Below 1 per cent we find sudden, in some cases very large variations. At about 0.05 per cent the optimum concentration is reached. With increasing dilution the speed of digestion is again reduced. In some cases a distinct inhibition is apparent at very great dilutions, in others however the extreme dilutions used are

practically like water. That bile salts do not seriously hinder the reaction is evident from those digestions where the bile salts were very concentrated. At least what inhibitory effect the salts may have at small dilutions is more than counterbalanced by their accelerating influence upon the reaction.

Another digestion series was undertaken to determine more exactly the nature of the reaction curve between the critical concentrations. Juice II was used, diluted 1:10. A final volume of 15 cc. was used in each case.

TABLE IX.

Ethyl Butyrate.

NO.	CC. BILE ADDED.	PER CENT BILE SOLUTION ADDED.	GRAMS BILE SALT ADDED.	FINAL CONCENTRATION PER CENT.	DIGESTION.
1.....	5	1.	0.05	0.333	6.7
2.....	4	1.	0.04	0.266	6.8
3.....	3	1.	0.03	0.200	7.4
4.....	5	0.5	0.025	0.166	8.7
5.....	4	0.5	0.020	0.133	10.0
6.....	3	0.5	0.015	0.100	12.0
7.....	2	0.5	0.010	0.066	14.0
8.....	5	0.1	0.005	0.033	18.0
9.....	4	0.1	0.004	0.026	20.0
10.....	3	0.1	0.003	0.020	20.0
11.....	5	0.05	0.0025	0.016	19.0
12.....	4	0.05	0.0020	0.013	19.0
13.....	3	0.05	0.0015	0.010	18.0
14.....	2	0.05	0.0010	0.006	17.0
15.....	1	0.05	0.0005	0.003	14.0

This table shows in a still more striking manner the double effect of bile salts upon the digestion of ethyl butyrate. There is positive inhibition above a percentage content of 0.066, while even above 0.026 inhibition is already in progress. Just what the mechanism of inhibition is we are in doubt. It may be due to a precipitating effect of bile acids upon the enzyme. Bile acids are known to precipitate proteins in general, and in this digestion the butyric acid liberated is probably strong enough to cause a certain amount of dissociation of the bile salts with a consequent effect of the bile acids on the enzyme. Such an effect would not be evident where a weaker acid or one less soluble was liberated,

as in the digestion of olive oil. Furthermore the addition of more bile salts to such a solution would finally tend to protect the enzyme. The effective action of an acid is as a rule diminished by the presence of large amounts of its salts. Hence in the former tables an excess of bile salts over 0.333 per cent diminished the inhibitory effect.

Turning from the effects of bile salts we shall present briefly data showing the alterations of the reaction by a variety of substances.

Dilution with water and salt solution. It had been noticed early in the experiment that undiluted juice acted toward ethyl butyrate less rapidly than one diluted 1:10. Accordingly digestions were made to determine the effect of regular dilution with water and with an electrolyte like common salt which is regularly present in the actual digestions *in vivo*. The effects are evidently quite different. In the case of salt, any acceleration due to dilution is masked by the inhibitory action of the chloride. Water alone accelerates up to a certain dilution—perhaps by allowing more of the zymolite to be in solution. Beyond that, dilution of the enzyme becomes a more potent factor. Balance between these opposite tendencies is reached at a dilution of about 1:20.

TABLE X.

One cc. juice + 10 drops ester.

NO.	WATER.	DIGESTION.	NO.	PER CENT SALT PRESENT.	VOLUME.	DIGESTION.
	cc.				*cc.*	
1.....	0	6.30	1.....	1.0	5	6.80
2.....	5	8.20	2.....	2.5	10	5.25
3.....	10	9.75	3.....	3.3	15	5.30
4.....	15	10.25	4.....	4.–	20	5.25
5.....	20	10.20	5.....	4.	25	4.20
6.....	25	8.20	6.....	4.1	30	4.20
7.....	30	6.75	7.....	4.3	35	4.30
8.....	35	2.90				
9.....	40	1.45				

Sodium hydroxide. It was believed that the inhibition exercised in a previous digestion by a commercial soap (Table VI),

might be due to the free sodium hydroxide. The following table shows that the effect of the strong base is a marked one, and that it affects both digestions alike. It may be therefore that in this case the action is directly upon the enzyme and that a certain amount of the hydroxide is necessary to completely inhibit the reaction. In almost every other case the nature of the zymolite has been one of the determining factors in the reaction, so that a substance which is found to inhibit the digestion of ethyl butyrate does not necessarily exercise the same action upon a digestion of olive oil.

TABLE XI.

NO.	NaOH CONCENTRATION.	ETHYL BUTYRATE DIGESTION.	OLIVE OIL DIGESTION.
1	$\frac{N}{30}$	0.0	0.0
2	$\frac{N}{60}$	0.0	0.0
3	$\frac{N}{300}$	0.55	0.0
4	$\frac{N}{3000}$	3.20	7.1
5	$\frac{N}{30000}$	2.90	8.70
6	water	3.10	7.40

Alcohol. Ethyl alcohol was found to exert a much more profound effect upon the digestion of ethyl butyrate than upon olive oil. Since alcohol is one of the decomposition products of the ethyl butyrate it is to be expected that its presence will influence the equilibrium finally attained in such a reaction. Its relation to the digestion of olive oil is obviously quite a different one since it is not liberated in the course of the digestion of

TABLE XII.

NO.	PER CENT ALCOHOL PRESENT.	ETHYL BUTYRATE. DIGESTION.	NO.	PER CENT ALCOHOL PRESENT.	OLIVE OIL DIGESTION.
1	31.	0.00	8	31.7	5.30
2	16.	4.30	9	23.3	6.05
3	9.	7.90	10	16.7	6.85
4	4.	9.00	11	6.6	7.20
5	0.9	10.00	12	3.3	8.40
6	0.4	10.40	13	0.33	9.85
7	0.0	10.20	14	0.0	7.85

triolein. It will be noticed that the effect of small amounts seems to be one of acceleration, in the case of olive oil, and this acceleration has been explained by some authors as an indication of a proenzyme present which is activated by the alcohol. We shall refer to this point again later for discussion.

Copper. The salts of copper present some interesting data. It was found in a preliminary digestion of ethyl butyrate and olive oil that much more pronounced inhibition was exerted in the case of the former ester. In each a rather sudden change from total inhibition to a rather slight inhibition was observed. Further digestions made this point clearly apparent. The two following tables show the effect of a copper sulphate solution.

In each digestion 1 cc. of Juice XVII was used, 10 cc. water, and 20 drops of the zymolite to be digested. The copper solution was added before the esters so that the action of the salt upon the enzyme itself could go on without modification at first. Corrections were made by means of blank digestions, to determine the amount of hydroxide necessary to precipitate the copper and give a distinct alkalinity to phenolphthalein.

TABLE XIII.

Olive Oil.

NO.	PER CENT $CuSO_4$ IN DIGESTION.	CORRECTION.	KOH.	DIGESTION.
1	0.0	0	10.50	10.50
2	0.1	1.50	1.50	0.00
3	0.09	1.30	0.80	0.00
4	0.08	1.25	0.50	0.00
5	0.07	1.20	0.80	0.00
6	0.06	0.90	4.25	3.35
7	0.05	0.75	7.95	7.25
8	0.04	0.60	10.65	10.05
9	0.03	0.50	9.85	9.35
10	0.02	0.30	11.85	11.55
11	0.01	0.15	10.20	10.05
12	0.001		10.20	10.20

TABLE XIV.

Ethyl Butyrate.

NO.	PER CENT $CuSO_4$ IN DIGESTION.	CORRECTION.	KOH.	DIGESTION.
1	0	0	3.05	3.05
2	0.1	1.50	1.20	0.00
3	0.07	0.90	0.80	0.00
4	0.05	0.75	0.70	0.00
5	0.02	0.30	0.40	0.10
6	0.01	0.15	0.20	0.05
7	0.007	0.09	0.20	0.11
8	0.005	0.07	0.05	0.00
9	0.002	0.03	0.25	0.22
10	0.001	0	1.20	1.20

From the two tables it will be seen that the digestion of olive oil begins appreciably in a solution containing 0.06 per cent of the copper salt. When ethyl butyrate is the ester acted upon digestion does not begin till a dilution of 0.002 per cent is reached. The inhibitory action of the copper is then thirty times greater in the digestion of ethyl butyrate than it is in the digestion of olive oil.

In the digestions cited the copper salt was readily soluble in the enzyme solution, insoluble in the zymolite. We have reversed these conditions by dissolving copper oleate in the olive oil and carried on the digestions for comparison. As might be expected the effect of the oleate is much less than that of the sulphate, and instead of showing a marked change from inhibition to digestion at a definite concentration, the oleate effects the reaction in a uniform manner. The greater the concentration of the copper salt in the zymolite the greater the inhibition. It seems probable that the sudden change in the copper sulphate series corresponds to the point at which all the copper is precipitated as carbonate or protein combination, or both.

To one series a 1 per cent copper sulphate solution was added in the amount tabulated, in the other copper oleate dissolved in toluol. Toluol itself we have shown to have no effect upon digestion of olive oil. The figures have been corrected for the precipitation of copper.

TABLE XV.

NO.	$CuSO_4$ SOL. 1 PER CENT, CC. ADDED.	DIGESTION.	COPPER OLEATE 1 PER CENT SOL. CC. ADDED.	DIGESTION.
1.....	1.0	0.00	1.0	1.20
2.....	0.5	0.25	0.5	2.80
3.....	0.1	8.00	0.1	6.20
4.....	0.05	7.70	0.05	7.20
5.....	0.01	7.40	0.01	7.10
6.....	0.0	7.35	0.0	7.35

Manganese. Since the salts of manganese have recently been shown to exercise accelerating effects on quite a number of enzymic reactions their action upon lipolysis has been examined. In our experiments we have failed to discover any true acceleration of the process, but we have found an effect closely allied. At a certain concentration the digestion proceeds normally—as though in a water solution. If the concentration of the manganese be altered in either sense inhibition of the lipolysis is the result. At a certain concentration therefore we have optimum digestion—representing a balance in all probability between two opposite tendencies. Diverging from this concentration alters the balance.

Tin. It has been found by Mr. Downing working in this laboratory that the salts of tin present much the same picture as the solution of manganous sulphate, except that at certain concentrations the tin salts show active accelerating effects. Since the results of his work will be presented in detail elsewhere, his figures will not appear in this paper.

In looking over the data presented in the tables just preceding one must be struck by the great variety of effects produced. Some substances react alike toward the digestion of either ester, others inhibit one strongly without great effect on the other, still others accelerate the digestion of one but not the other. Taken separately such results are liable to misinterpretations. For example the effects of alcohol on the digestion of olive oil led Gizelt[1] to the belief that a proenzyme exists in the secretion which alcohol activates. Using the same reasoning water must activate a proenzyme for the digestion of ethyl butyrate; bile salts, in certain concentrations do the same; sodium chloride solutions

[1] *Arch. f. d. ges. Physiol.*, cxi, p. 620.

TABLE XVI.

Olive Oil.

NO.	$\frac{N}{10}$ $MnSO_4$ ADDED.	FINAL CONCENTRATION.	KOH.	CORRECTION.	DIGESTION.
	cc.				
1.....	5	$\frac{N}{20}$	10.00	control	no enzyme added
2.....	5	$\frac{N}{20}$	13.80	10.00	3.80
3.....	4	$\frac{N}{25}$	12.00	8.00	4.00
4.....	3	$\frac{N}{33}$	11.20	6.00	5.20
5.....	2	$\frac{N}{50}$	10.80	4.00	6.80
6.....	1	$\frac{N}{100}$	9.20	2.00	7.20
7.....	0.7	$\frac{N}{143}$	6.80	1.40	5.40
8.....	0.5	$\frac{N}{200}$	5.70	1.00	4.70
9.....	0.2	$\frac{N}{500}$	4.80	0.40	4.40
10.....	0.1	$\frac{N}{1000}$	4.85	0.20	4.65
11.....	0.05	$\frac{N}{2000}$	5.16	0.10	5.06
12.....	0.00	0	7.20		7.20
		Ethyl Butyrate.			
1.....	5	$\frac{N}{20}$	8.8	8.1	0.70
2.....	4	$\frac{N}{25}$	8.6	6.42	2.50
3.....	3	$\frac{N}{33}$	7.7	4.86	2.90
4.....	2	$\frac{N}{50}$	7.4	3.24	4.20
5.....	1	$\frac{N}{100}$	4.6	1.60	3.00
6.....	0.5	$\frac{N}{200}$	4.45	0.80	3.60
7.....	0.2	$\frac{N}{500}$	1.45	0.30	1.10
8.....	0.1	$\frac{N}{1000}$	2.00	0.15	1.85
9.....	0.0	0	3.7	0.0	3.78

do not; tin salts activate while copper salts do not, and so on. It seems much more reasonable to conclude that the lipase is already active as secreted, but that all of these substances modify in one way or another the speed and extent of digestion. Indeed if this argument for activation be used we should have to suppose that activated tryptic and peptic digestions may be further activated, for a number of substances are known to accelerate proteolytic digestions. Amylolytic digestions proceed more rapidly under certain conditions—as for example in the presence of small amounts of manganese and iron—and we should have to assume a similar activation there. The evidence is obviously

not sufficient in any of these examples for an activation of a pro-enzyme. On the other hand it points strongly to a modification of the speed of reaction and of the final point of equilibrium.

Again many of the preceding tables may be interpreted to indicate the presence of two distinct enzymes—one which splits triolein the other ethyl butyrate. Thus copper sulphate affects the digestion of ethyl butyrate very powerfully, while its action toward olive oil is comparatively slight. Alcohol acts in the same way. Bile salts behave very differently in the two digestions. Right through the list of reagents tested, the usual result is different in the two digestions. It is not necessarily so however, as the experiment with sodium hydroxide has shown. But it is upon just such kind of evidence that many conclusions have been drawn as to the presence of two or more distinct enzymes in a solution. Hattori[1] for example has recently supported the early view of Pollack[2] that there was a separate gelatin-digesting enzyme in pancreatic juice distinct from trypsin. The digestion of gelatin is influenced differently from that of coagulated albumin by the addition of various salts to the mixture, and a glutinase is therefore assumed to be present. It seems to us that there is something more than a matter of definition involved here. If we assume that the enzyme proper is the colloidal, thermolabile substance present in the solution which acts as the catalytic agent in conjunction with a great variety of salts, both organic and inorganic, then it seems unnecessary to postulate a distinct enzymatic entity for every catalytic activity of this complicated mixture. The factors that may be considered accidental are frequently of great importance in an enzyme reaction. For example the loss of amylolytic power of pancreatic juice subjected to dialysis was shown by Bierry and Giacosa[3] to be due to the removal of the chlorides. They claim that prolonged dialysis removes the characteristic activity of the amylopsin completely, but that the addition of sodium chloride for example, restores it. While our own results in attempting to verify these reports have not been so striking they have at least confirmed the general

[1] *Arch. internat. de pharmacodyn.*, xviii, p. 255.

[2] *Beitr. z. chem. Physiol. u. Path., vi*, p. 95; also *Arch. f. Verdauungskrankheiten*, xi, p. 362, cited from *Biochem. Centralbl.*, iv, p. 394.

[3] *Compt. rend. soc. biol.* lxii, p. 432.

statement that the chlorides always present in the normal juice are important factors in the speed of the reaction. We have been unable to completely suspend amylolytic activity by dialysis, though we have greatly diminished it, and have found it again increased by the addition of sodium chloride. Magnus[1] found that liver lipase could be rendered totally inactive by dialysis, but that the activity could be restored by returning the dialysate. Loevenhart[2] confirmed this and found the necessary substances to be bile salts. Without their presence the amyl salicylate could not be hydrolyzed. If even the fundamental reactions of the thermolabile colloid we call the enzyme are quite dependent upon the presence of other substances, it is not at all surprising that other manifestations of the catalytic activity—like the splitting of various unphysiological esters by lipase, and the digestion of polypeptides or gelatin by trypsin—should also be modified by the presence or absence of other compounds. And since the zymolite itself probably exerts its own characteristic influence on the reaction, it is to be expected that an added substance like alcohol or a copper salt or sodium hydroxide should effect the reaction differently in the presence of different zymolites. It may even be suggested here that the pancreatic maltase which manifests its action only in a neutral or faintly acid medium—according to the investigations of Terroine and Bierry[3]—and which is not found in the normal juice because of its alkalinity, is but another expression of the same enzyme which normally converts starch into maltose. At least where one type of catalytic activity is always accompanied by another—if the environment is such as to allow the second to show itself—and no definite separation can be effected, the application of names indicative of activity, seems to us to involve confusion of ideas. When a real separation of enzymes, or a very definite distinction, is apparent there is some point in assigning new names. We believe that *pepsin* and *trypsin* are names designating two very distinct catalytic agents; we do not believe that the name *glutinase* as indicative of a catalytic agent distinct from trypsin is any more justifi-

[1] *Zeitschr. f. physiol. Chem.*, xlii, p. 149.
[2] *This Journal*, ii, p. 391.
[3] *Compt. rend. de l'acad. des sci.*, cxli, p. 146.

able than would be the application of a list of names designating the various ester-splitting powers of lipase.

Progress of digestion. In all the figures tabulated thus far a single point in the digestion has been represented. It is obvious that while comparisons of such figures indicate cross sections of digestions as it were, at the given time, little appreciation of the steps of the reaction can be gained from them. For example in table VI it was shown that the effect of bile salts in the digestion of olive oil could be duplicated by agitation and emulsification in solutions lacking the bile. In that particular experiment and at that particular time the cross sections were alike, but it cannot be concluded that the reactions have been alike all through, nor that they will be alike at a later period. The initial stages of one may have been rapid, but later compensated by a retardation, so that the curves representing digestions would be seen to cross. We have therefore concluded to examine these reactions in progress; to discover in detail the effect of bile salts, of the amount of ester, and of the amount of enzyme.

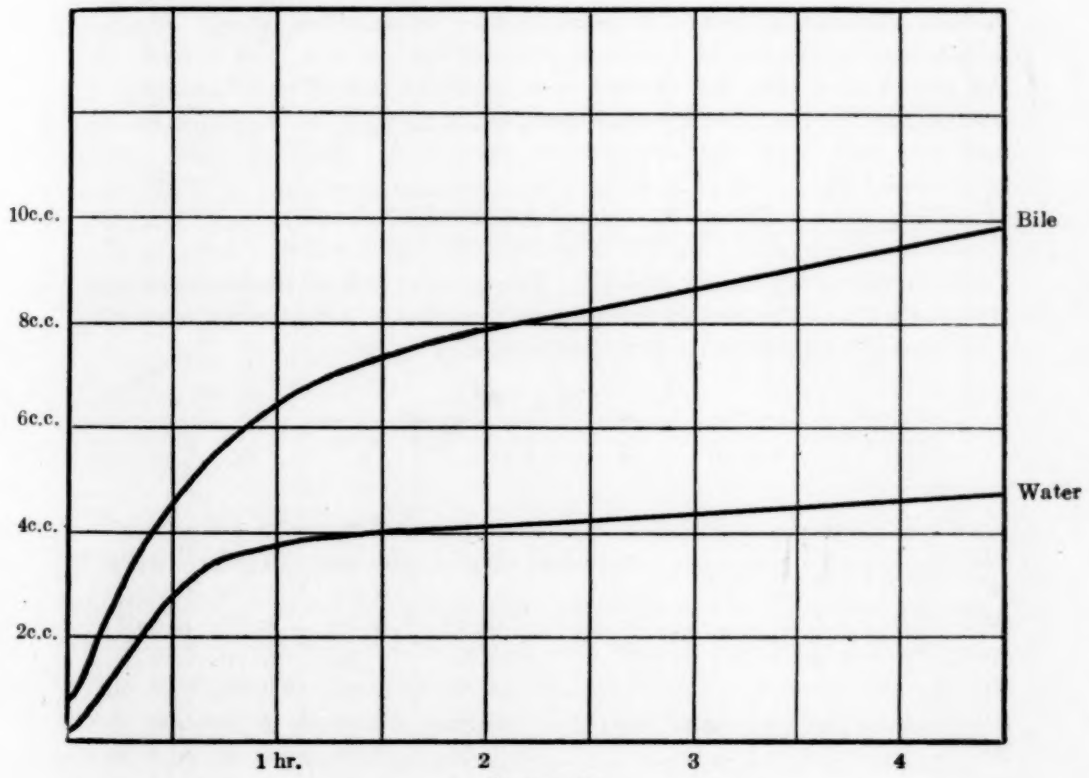
A preliminary experiment showed that where two digestions were carried on side by side with constant agitation, one diluted with bile salt solution, the other with water, the former proceeded faster and farther than the latter. One such experiment may be used to illustrate.

Two glass stoppered bottles of 500 cc. capacity were used, 20 cc. of Juice XVII added to each and 200 cc. of water or 2 per cent bile solution. Ten cc. of olive oil were added to each, the bottles shaken vigorously and a 10 cc. sample taken from each. At intervals of time shown in the table 10 cc. samples were removed and titrated with alcoholic potash. The bottles were shaken at regular intervals alike to insure equal emulsification if such were possible. It was evident at the beginning that the bile mixture contained by far the better emulsion. Later when soaps had formed it was impossible to determine which had the finer emulsion, but it seems reasonable to suppose the advantage continued to be with the bile solution. If this were the case the bile mixtures should continually present a larger surface of zymolite to the enzyme, and hence an effect of mass be produced. The figures illustrate the great initial speed of the reaction. At the end of an hour practically 50 per cent of the fatty acid liberated in the 10 hour period has been set free; 25 per cent in the first 15 minutes. Plotting the reaction curves, the bile mixture resembles the reaction in water except that it attains a higher level of equilibrium, The curves are identical in form. The effect of bile then may be simply one

of emulsification, or it may also exert a protective function over the enzyme in the presence of the fatty acid liberated.

TABLE XVII.

TIME.	0.	5′.	10′.	15′.	20′.	25′.	30′.	40′.	50′.	1 hr.	2.	3.	10.	48.
Water...	0.2	0.55	1.35	1.60	1.60	2.00	3.00	3.40	3.50	3.80	4.10	4.1	6.9	7.0
Bile.....	0.75	1.35	2.20	3.40	3.50	4.00	4.50	5.30	5.95	6.50	7.90		13.6	13.7



CURVE I.

The final digestion of the oil in water is seen to be little more than half that in the presence of bile salts. It was thought therefore that by doubling the amount of zymolite in the mixture an approximation to doubling the effective mass might also be assumed where the digestions are subjected to continual shaking at least. If the effect of bile is purely physical then digestion should proceed in this case about as far as in the bile mixture above. If however the salts exert a protective action as well, such a comparative digestion should bring it out. The effect of the mass of enzyme was also determined in this series.

The digestions were carried out as before. The digestive mixtures are shown in the table:

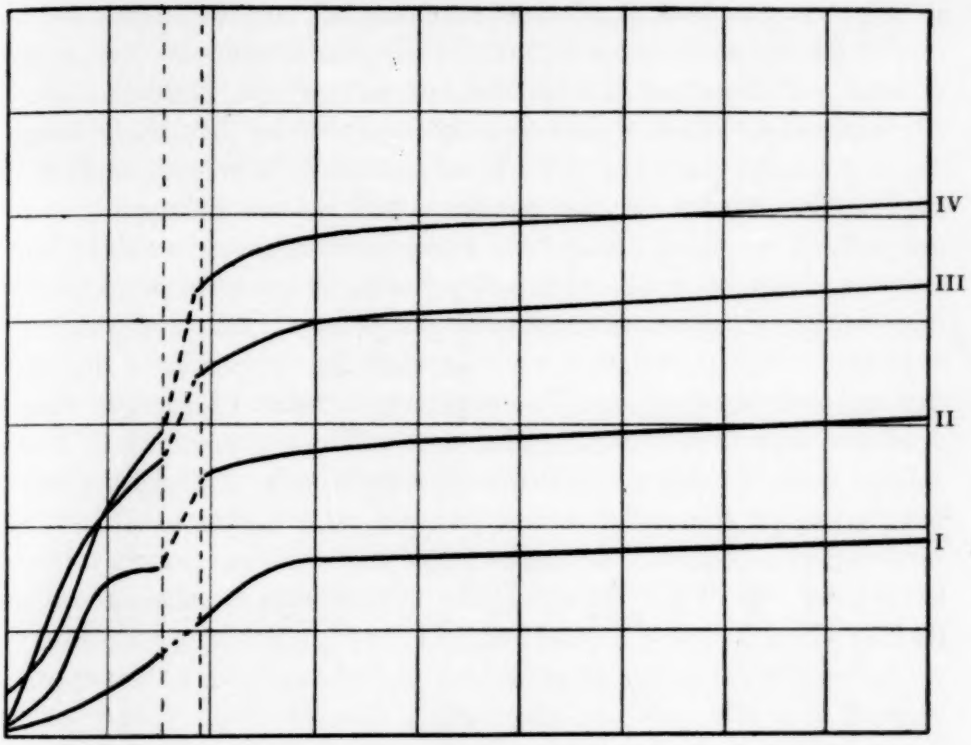
TABLE XVIII.

I	5 cc. juice	200 cc. water	10 cc. olive oil
II	10 " "	200 " "	10 " " "
III	10 " "	200 " "	20 " " "
IV	10 " "	200 " bile	10 " " "

When the mixtures were ready the oil was added rapidly and the four bottles thoroughly shaken in order and 10 cc. samples taken. Shaking and sampling proceeded every 10 minutes for an hour and a half. At the end of that time the bottles were suddenly chilled to 0° and kept so overnight. In the morning they were again warmed up together, shaken and sampled, and the experiment continued. Samples were taken occasionally for two days to determine approximately what the final point of equilibrium would be. In I the point of equilibrium was early attained, in the others digestion was still proceeding at the end of two days—slowly in II, more rapidly in III and IV. The plotted curves indicate the relative positions of the four digestions and the effect of bile salts, zymolite, and mass of enzyme better than the tabulated figures.

TABLE XIX.

TIME.	I.	II.	III.	IV.
0	0.1	0.2	0.1	0.8
10	0.2	0.45	1.15	1.20
20	.	0.50	1.80	1.50
30	0.30	0.90	2.70	2.30
40	0.40	1.80	3.70	3.25
50	0.60	2.40	4.00	3.70
60	0.80	3.00	4.30	4.25
70	1.05	3.10	4.60	4.90
80	1.05	3.00	5.00	5.35
Stood cold over night.				
0	2.10	4.50	6.90	8.55
10	2.40	5.10	7.05	8.85
30	3.30	5.25	7.90	9.30
60	3.30	5.25	7.60	9.50
2 hrs.	3.45	5.75	8.10	9.70
5 "	3.30	6.00	8.40	10.40
7 "	3.80	6.00	8.60	10.10
48 "	3.80	6.40	10.00	12.25



1 hr. CURVE II

Examining the tables and curves the following points are of interest: No. I shows clearly the slow initial steps of the reaction, the gradual increase of speed and the early arrival at the plateau region, where digestion has practically reached equilibrium at the end of four hours. The curve is characteristic of an autocatalytic reaction and in this case the autocatalysis may depend upon the formation of soaps, thus increasing the emulsion and speed during the first few periods of the digestion. No. II shows the same typical curve but the autoacceleration proceeds much more rapidly and the plateau is much higher than in No. I. While the degree of digestion is not exactly proportional to the mass of the enzyme it evidently bears a relation somewhat suggestive of such proportionality. Hedin[1] found that the *amount of enzyme* times the *time* of digestion was a constant quantity ($P \times T = K$). In the table above No I should require twice the time to liberate a given amount of acid compared with No. II. In a few instances

[1] *Zeitschr. f. physiol. Chem.*, lvii, p. 468.

the figures agree with this hypothesis, but not very exactly. Nos. II and III illustrate the effect of doubling the amount of zymolite present. It does not double the amount of acid liberated in a given time but it very considerably increases it. As the curves show, a smaller per cent of the total available fatty acid is liberated in III than in IV; though the actual amount is larger. The neutral oil may exert a certain protective influence so that the enzyme is not inhibited so soon by the fatty acid liberated, or it may increase digestion simply by its greater mass and surface exposed. It is interesting to note that the digestion of double the amount of oil—No. III— begins at a more rapid rate than does the digestion of the regular amount in the presence of bile salts. After the first hour however digestion in the presence of bile outstrips the other and continues to a higher level before reaching equilibrium. It seems to us that this fact points to the protective rôle of bile salts. If the emulsification were the only feature of its action we should expect its most striking exhibition at the very beginning of the reaction, for at that point the advantage of the bile mixture is greatest. Later when soaps have formed abundantly in all the digestions the advantage of the bile mixture would be proportionately less. We find however that No. IV lags very distinctly at first, but that later when the inhibiting effect of the fatty acids begins to be noticed in III and II the bile mixture continues for some time to increase its total acid content. At the end of forty-eight hours it contains nearly twice the amount of acid as No. II. Where emulsification is insured in all digestions by agitation, the protective effect of the bile salts over the enzyme seems to us to be their chief function in digestion. Where agitation is slight they must also facilitate through their emulsifying power.

In a single trial we have made with ethyl butyrate no such effect of the bile salts is apparent. The digestion in which bile was present seemed to have no advantage to speak of at first and later was distinctly outstripped by the water solution. Final equilibrium was practically the same. The figures are presented in Table XX.

The effect of temperature. The lipolysis of olive oil is one which lends itself readily to the study of the effect of temperature. While the hydrolysis of an ester is a simple monomolecular reaction,

there are factors involved in this case which may be expected to complicate the results considerably. A gradual rise in temperature from zero degrees to body temperature should be accompanied by an increased digestion following the law of van't Hoff; the speed of the hydrolysis should double as the result of a 10° rise of temperature, if the enzyme alone were concerned. But along with this increased speed of hydrolysis the mobility of the oil is also increased, leading to better emulsification perhaps and readier contact with the enzyme. This should result in a further acceleration of the reaction. On the other hand the solubility of oleic acid in water must be much greater at 40° than it is at zero. While we have not been able to find definite figures upon this point, the increasing solubility of the

TABLE XX.

TIME.	WATER.	BILE.	TIME.	WATER.	BILE.
0	0.0	0.20	3 hrs.	0.80	0.70
10 min.	0.05	0.20	3½	1.00	0.75
25	0.15	0.30	4	0.95	0.80
55	0.15	0.35	4½	0.95	0.82
85	0.35	0.45	5	0.95	0.80
2 hrs.	0.50	0.55	48	1.05	1.05
2½	0.65	0.65	72	1.10	1.00

lower fatty acids at higher temperatures offer an analogy in point. With increasing solubility and mobility the oleic acid will exert an increasing inhibitory and destructive effect upon the enzyme. Thus we have two opposing tendencies apparent in this reacting mixture making it improbable that even an approximation to van't Hoff's law will be found—except perhaps within certain narrow limits where a balance between factors may occur. A further complication exists as we raise the temperature toward the point at which the enzyme is destroyed—we are unaware of the effect of temperatures several degrees below this critical point. Even the agitation may produce a different effect at one temperature from that at another.

This brings us to one of the most interesting points brought out in these experiments—*the inhibitory effect of excessive agita-*

tion. We assumed that increased agitation would produce better contact of enzyme and oil, better emulsification, better distribution of the digestion products, and thus better digestion. Up to a certain point this holds true—a digestion subjected to moderate shaking proceeds more rapidly than one left undisturbed. When pushed to an extreme however, agitation was found to actually hinder the reaction. This may be due in this case to the diluting effect of the air bubbles continually enclosed in the mixture in great numbers and thus preventing contact of enzyme and zymolite, much as the addition of large amounts of any inert material might. It may be on the other hand that the air bubbles destroy the enzyme by coagulation—just as egg albumin is coagulated in beating. We have some evidence which at present inclines us to the view that the enzyme is itself destroyed slowly by agitation violent enough to produce frothing. Whatever the cause of this phenomenon may prove to be later, the fact remains that a digestion may be seriously inhibited in the later stages of the reaction, and the final point of equilibrium considerably depressed by too frequent shakings. In the earlier stages the reaction frequently shows equal or greater speed than one where the shaking is less vigorous. A typical case of this inhibition by agitation is presented in the table following.

The digestion bottles contained 100 cc. Juice XVII diluted 1:10, and 5 cc. of olive oil. Both bottles were shaken vigorously and samples taken. Thereafter No. 1 was kept in constant motion by a shaking machine. The stroke of the machine was about 5 inches at a speed of about 200 per minute. No. II was given 20 vigorous shakes at 2 minute intervals. Samples were removed for titration every 10 minutes.

TABLE XXI.

TIME.	0.	10′.	20′.	30′.	40′.	50′.	60′.	
No. 1	1.30	1.80	1.80	2.25	2.20	2.20	2.30	constant shaking
No. 2	1.10	2.00	2.60	2.80	3.10	3.30	3.55	20 shakes in 2 min.

CURVE III.

As will be seen the two digestions are practically alike for 10 minutes. At the end of that time the amount of soaps produced in the mixtures is sufficient to give considerable frothing. In the bottle kept continually in motion the effect is one of a permanent admixture of a large amount of air. In the bottle shaken at intervals the foam has a chance to rise and disappear.

With these results before us it was evident that a uniform procedure of shaking at stated intervals should be adopted for the series of digestions designed to test the effect of temperature. Table XXII presents the results obtained in three series of such digestions.

The shaking machine was so arranged that a number of bottles could be fastened to the piston and moved up and down in a tank of water where the temperature could be kept constant. The bottles containing the enzyme solution were placed in the tank in position and agitated for ten minutes to insure correspondence with the temperature of the bath. The oil was then measured in and the motor started. After each 15 seconds of vigorous shaking the digestions were allowed to stand quiet for 2 minutes. As before samples were removed every 10 minutes and

titrated. Each bottle contained 100 cc. of an active juice diluted 1:10 and 5 cc. oil added. The digestions were usually continued for an hour—in some cases longer. As a rule the digestions approach the plateau level by the end of an hour.

TABLE XXII.

Series I, Juice XVII.

TEMP. DEGREES.	10.	20.	30.	40.	50.	60.	70.	80.	90 Min
0	1.10	1.70	2.30	2.30	2.40	2.80	2.65		2.80*
10	1.30	1.95	2.30	3.00	3.75	4.05	4.50		4.75*
20	1.85	3.20	4.00	4.65	5.15	5.45	5.90		5.90*

Series II, Juice XVII.

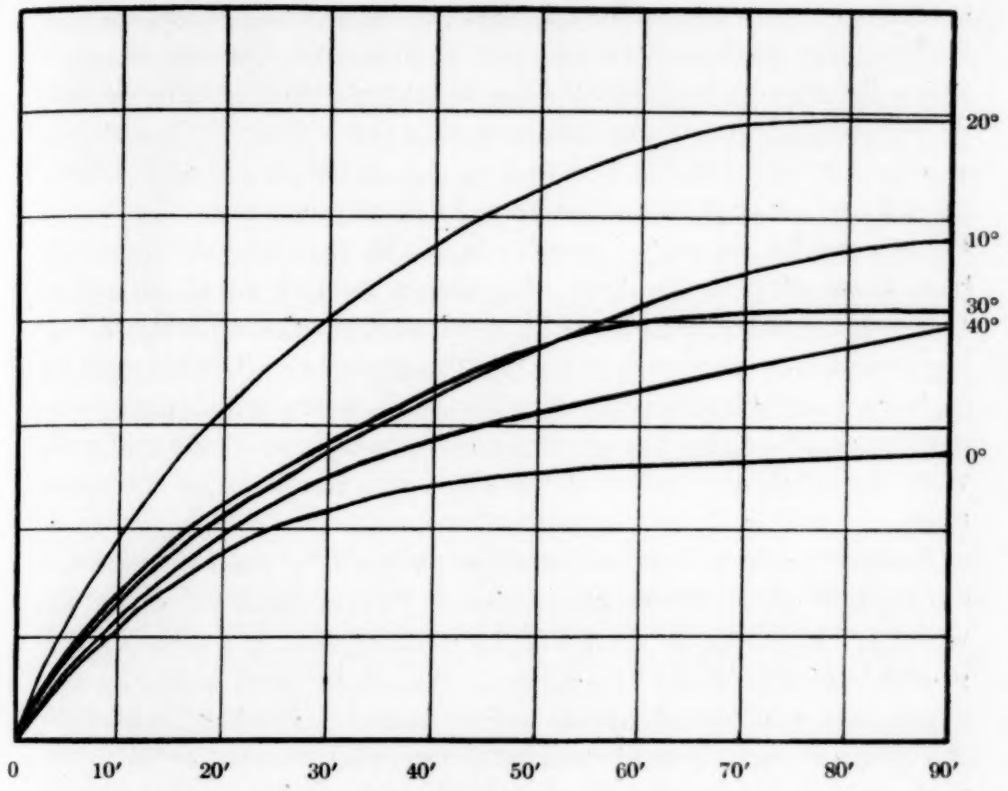
20	2.00	3.20	3.60	4.60	4.80	5.45	6.00		6.00
30	1.30	2.80	2.80	3.10	3.70	4.00	4.05		4.15*
40	1.10	2.00	2.60	2.80	3.10	3.30	3.55		4.00*

Series III, Juice XII.

0	1.00	1.70	2.15	3.70	3.80	5.50			
10	1.10	2.15	3.50	4.05	5.10	6.19			
20	1.30	2.40	3.90	5.50	5.00	5.50	6.00		
40	1.90	3.10	3.85	3.85	4.60	4.80	5.00		
40	2.05	3.10	4.05	4.15	5.00	4.80	5.30		
50	0.75	2.00	2.30	2.70	2.90	3.00	3.10		
60	0.00	0.00	0.00	.	.	.	0.00		

Note. Cf. Curve iii.

From the table, Series I and II, it is evident that the agreement between duplicates is quite good. The digestion, emulsification, and sampling are practically uniform and to be depended upon in the large. Individual samples are frequently high or low, but in general where a number of successive samples are taken the figures should represent pretty accurately the amount of acid present. Curves drawn from these figures should give a still more accurate conception of the reactions.



CURVE IV

In Series I and II the rather striking fact is brought out that digestion at 20° is the most rapid and the most complete. In Series III where a different juice is used the digestion at zero is as complete as one at twenty, though the initial stages are not so rapid. At 10° and 20° digestion in III is at its best—more rapid in the first stages at the higher temperature, but less complete. Inhibition in all the digestions is manifested earlier in the higher temperatures. This we believe is expressive of the increasing solubility of the oleic acid in warm water. The acid is thus enabled to inhibit or destroy the enzyme better in those solutions where it is most readily soluble. Thus the earlier stages of digestion are accelerated by elevation of the temperature, up to 40°; the later stages indicate the retardation produced by increasing amounts of acid in solution.

In reactions subjected to this variable factor we should hardly expect agreement with the law of van't Hoff except in certain

portions of the series. In selected portions of the reaction the figures show conformity to the law mentioned. Thus in the first series digestion is doubled by a rise in temperature from 10° to 20°. At 20° we have for example the same amount of acid liberated in 30 minutes as is liberated in 60 at 10°. Between 20° and 30° we do not find an approximation to the law at any point. In Series III the results are more irregular for some reason and show still less conformity to the laws of a simple hydrolysis of an ester. It is evident that the nature of the substances present in the different samples of juice may influence the reaction. The amount of carbonate and bicarbonate will play an obvious part in complicating the reaction, for the presence of uncombined alkali will prevent the inhibitory effect of the oleic acid liberated in the reaction.

A question which naturally suggests itself by the temperature curves is whether the enzyme itself is weakened at 30° or 40° or whether the effect is produced by the inhibitory action of the products of digestion. In Series I and II the action of the enzyme seems to diminish after 20° is passed. Possibly in sample XVII the enzyme is weakened by temperatures of 30° or 40°. In Series III, however, the digestions increase in their initial speeds till 40° is passed. At 50° the enzyme is clearly weakened. The initial stages of the digestion decrease in speed. At 60° the enzyme is completely destroyed.

The tables of reaction figures under discussion allow of further examination for reaction constants. Kanitz[1] and Engel[2] have both shown that at a given temperature, with equal amounts of enzyme present the amount of acid liberated bears a constant relation to the time of digestion. The expression for this relation is $a/\sqrt{t} = K$ where a = the amount of acid liberated, and t = time of the reaction. Our own figures bear out this relation as will be seen in the following tables. It seems obvious however that such an expression can only hold good during the active digestion stage. As soon as equilibrium is approximated the speed of the reaction decreases and finally approaches zero as a limit. In other words after the curve has reached the plateau level a

[1] *Zeitschr. f. physiol. Chem.* xlvi, p. 482.
[2] *Beitr. z. chem. Physiol. u. Path.*, vii, p. 77.

will become more and more a constant quantity, while t continues to increase. As the reaction becomes stationary or balanced the constant K will therefore decrease in value—will no longer be a constant. Furthermore it seems to us likely that K will not be a constant in the initial stages of the reaction where the products of digestion exert an autocatalytic effect. While we have no complete set of results to illustrate this hypothesis, we can obtain a partial confirmation from the figures of Table XIX. Calculations are made with the same expression and it will be evident that the value of K is not a constant one till the reaction has progressed for 40 minutes—that is till the effect of active autocatalysis has been passed—expressed on the curve by a uniform upward direction.

The calculations of reaction constants are based upon the tables of the text already presented. The time t, and the amount of digestion in that time, a, will be given however for clarity, with the calculated value K. Table XXIII is based upon XXII. The effect of temperature in modifying the value of K is therefore exhibited here.

In the following table (XXIV) the calculations of K are based upon the figures of Table XIX. In collecting the data for that table the experiment had to be discontinued after 80 minutes had elapsed. The bottles were chilled to 0°, and allowed to stand overnight. The next morning the experiment was resumed and readings taken during 7 hours of that day. By this time the digestions were approaching equilibrium and change from hour to hour was very slow even with frequent shaking. The figures for 48 hours therefore will not be far from the figures which would have resulted if the digestions were shaken during the second night. During both days the bottles were shaken frequently and kept at the same temperature. In presenting the calculations below it is evident that some allowance for the digestion which went on during the first night must be made. The actual time is of no significance here for the reason that the digestions were proceeding under conditions entirely different from normal. We have assumed therefore that the value for K which was found at the end of the 80 minute period, is also the value for K when normal conditions were restored again the next morning. Making this assumption we have then calculated the time in which this same digestion would have been accomplished if the conditions of temperature and shaking had remained normal. While the values for K obtained in the subsequent portion of the digestion must be subject to whatever error was involved in the assumption made, it seems likely that these values are not far from being correct. We should not look for a sudden change in value of K anywhere in the digestion, and it will be evident in the tables that K remains practically a constant until 5 hours of the second day had elapsed.

TABLE XXIII.

TIME.	0°.		10°.		20°.	
	a.	*K.*	*a.*	*K.*	*a.*	*K.*
10′	1.10	0.348	1.30	0.411	1.85	0.585
20′	1.70	0.380	1.95	0.436	3.20	0.716
30′	2.30	0.420	2.30	0.420	4.00	0.730
40′	2.30	0.364	3.00	0.474	4.65	0.735
50′	2.40	0.339	3.75	0.530	5.15	0.728
60′	2.80	0.361	4.05	0.523	5.45	0.704
70′	2.65	0.317	4.50	0.538	5.90	0.705
90′	2.80	0.295	4.75	0.501	5.90	0.622
	20°		30°.		40°.	
10′	2.00	0.632	1.30	0.411	1.10	0.348
20′	3.20	0.716	2.80	0.626	2.00	0.447
30′	3.60	0.657	2.80	0.511	2.60	0.475
40′	4.60	0.727	3.10	0.490	2.80	0.443
50′	4.80	0.679	3.70	0.523	3.10	0.438
60′	5.45	0.704	4.00	0.516	3.30	0.426
70′	6.00	0.717	4.05	0.484	3.55	0.424
90′	6.00	0.632	4.15	0.437	4.00	0.422
	0°.		10°.		20°,	
10′	1.00	0.316	1.10	0.348	1.30	0.411
20′	1.70	0.380	2.15	0.680	2.40	0.537
30′	2.15	0.393	3.50	0.783	3.90	0.711
40′	3.70	0.585	4.05	0.740	5.50	0.870
50′	3.80	0.537	5.10	0.806	5.00	0.707
60′	5.50	0.710	6.19	0.875	5.50	0.710
70′	.	.	.	.	6.00	0.717
	30°.		40°.		50°.	
10′	1.90	0.601	2.05	0.648	0.75	0.237
20′	3.10	0.693	3.10	0.693	2.00	0.447
30′	3.85	0.703	4.05	0.740	2.30	0.420
40′	3.85	0.609	4.15	0.656	2.70	0.427
50′	4.60	0.651	5.00	0.707	2.90	0.410
60′	4.80	0.620	4.80	0.620	3.00	0.387
70′	5.00	0.598	5.30	0.633	3.10	0.370

as was said before, when the digestion approaches equilibrium K will no longer be a constant but will steadily diminish. It is obvious that since the digestions proceed differently during the night, the time to be allowed will not be the same for all.

TABLE XXIV.

I.			II.			III.			IV.		
TIME.	a.	K.	TIME.	a.	K.	TIME.	a.	K.	TIME.	a.	K.
10′	0.20	0.063	10′	0.45	0.14	10′	1.15	0.36	10′	1.20	0.38
20′	0.		20′	0.50	0.11	20′	1.80	0.40	20′	1.50	0.34
30′	0.30	0.055	30′	0.90	0.16	30′	2.70	0.49	30′	2.30	0.42
40′	0.40	0.063	40′	1.80	0.28	40′	3.70	0.59	40′	3.25	0.51
50′	0.60	0.085	50′	2.40	0.34	50′	4.00	0.57	50′	3.70	0.52
60′	0.80	0.103	60′	3.00	0.39	60′	4.30	0.56	60′	4.25	0.55
70′	1.05	0.125	70′	3.10	0.37	70′	4.60	0.55	70′	4.90	0.59
80′	1.05	0.117	80′	3.00	0.34	80′	5.00	0.56	80′	5.35	0.60
Time calculated for each, from the formula $a\sqrt{t} = K$; *K assumed constant.*											
320′	2.10	0.117	180′	4.50	0.34	152′	6.90	0.56	204′	8.55	0.60
330′	2.40	0.132	190′	5.10	0.37	162′	7.05	0.55	214′	8.85	0.61
350′	3.30	0.18	210′	5.25	0.36	182′	7.90	0.59	234′	9.30	0.61
380′	3.30	0.17	240′	5.25	0.34	212′	7.60	0.52	264′	9.50	0.59
440′	3.45	0.17	300′	5.75	0.33	272′	8.10	0.49	324′	9.70	0.54
620′	3.30	0.13	480′	6.00	0.27	452′	8.40	0.40	504′	10.40	0.46
740′	3.80	0.14	600′	6.00	0.25	572′	8.60	0.36	624′	10.10	0.40
2300′	3.80	0.07	3060′	6.40	0.12	3032′	10.0	00.18	3084′	12.25	0.22

The following table is based upon the figures of XVII and XXI. I represents a digestion in water, II in bile; III with intermittent shaking, IV with continuous.

TABLE XXV.

I.			II.			III.			IV.		
TIME.	*a.*	*K.*	TIME.	*a.*	*K.*	TIME.	*a.*	*K.*	TIME.	*a.*	*K.*
5′	0.55	0.24	5′	1.35	0.60	10′	2.00	0.63	10′	1.80	0.57
10′	1.35	0.43	10′	2.20	0.70	20′	2.60	0.58	20′	1.80	0.40
15′	1.60	0.41	15′	3.40	0.88	30′	2.80	0.51	30′	2.25	0.41
20′	1.60	0.36	20′	3.50	0.78	40′	3.10	0.49	40′	2.20	0.35
25′	2.00	0.40	25′	4.00	0.80	50′	3.30	0.47	50′	2.20	0.31
30′	3.00	0.55	30′	4.50	0.82	60′	3.55	0.46	60′	2.30	0.30
40′	3.40	0.54	40′	5.30	0.84						
60′	3.80	0.49	60′	6.50	0.84						
120′	4.10	0.37	120′	7.90	0.66						
180′	4.10	0.31	180′								
600′	6.90	0.28	600′	13.60	0.76						
2880′	7.00	0.13	2880′	13.70	0.48						

The final table on this point is calculated from one of a number of earlier experiments not reported in the paper. The digestion covered a two-day period; shaken often during the first 7 hours, and for 9 hours on the second day, 2 hours on the third. The digestion had therefore progressed so far that allowing the flask to stand unshaken overnight would produce little slowing of the reaction. That is so say, the amount of soaps present after the first few hours is sufficient to keep the emulsion for a long time, so that frequent or infrequent shaking makes little difference with the digestion. While the figures for the last three samples show considerable irregularity, they represent accurately enough the fact that the value of K diminishes as the reaction approximates a condition of equilibrium. They also show that the reaction starts slowly and gradually attains its maximum speed—in this case after 30 minutes of digestion. This is of course indicative of the autocatalytic nature of the reaction.

TABLE XXVI.

TIME.	*a.*	*K.*	TIME.	*a.*	*K.*	TIME.	*a.*	*K.*
15′	1.20	0.31	90′	8.15	0.86	360′	11.25	0.59
30′	3.50	0.64	105′	8.60	0.84	420′	11.20	0.55
45′	5.80	0.87	120′	8.70	0.80	1380′	11.50	0.31
60′	6.60	0.85	135′	9.10	0.78	1500′	13.80	0.36
75′	7.35	0.85	285′	10.50	0.62	2880′	12.60	0.19

SUMMARY.

The following points have been brought out in our examination of this human pancreatic juice:

(1) The specific gravity averages about 1010.

(2) Alkalinity is due to normal and bi-carbonates—chiefly the latter. The secretion varies from a $\frac{N}{20}$ to $\frac{N}{10}$ bicarbonate solution.

(3) No definite relation could be established between diet and enzyme content. The enzymes are found to vary considerably with the exception of amylopsin which appears to be more constant in amount from day to day. A slowly secreted juice is more concentrated in its solid content, its carbonates, and lipase.

(4) Rennin, invertase, and lactase were not found.

(5) Manganous sulphate was not found to accelerate starch digestion, nor was bile.

(6) Trypsin was found in about half the samples collected. Inactive samples could be readily activated with enterokinase prepared from the intestines of dogs and cats. Inactive samples became gradually active on standing, though saturated with toluol and in some cases chloroform.

(7) The lipolysis of olive oil and ethyl butyrate vary in parallel directions in the different samples. We believe a single enzyme accomplishes both digestions.

(8) An examination of the effect of various substances upon the digestions of olive oil and ethyl butyrate shows that certain compounds affect both alike, while the majority influence the hydrolysis of one ester more profoundly than the other. The zymolite exercises an important effect in the modification of the reaction produced by the added compound. A notable example of this point is the action of bile salts in the two digestions. Upon the digestion of olive oil it exerts a twofold influence: it assists emulsification, and it protects the enzyme from the inhibitory action of the acid liberated. The effect upon the digestion of ethyl butyrate is much more complex. It inhibits or it accelerates, depending upon its concentration.

(9) The hydrolysis of olive oil proceeds as an autocatalytic reaction at first. This may be due to the formation of soaps and consequent emulsifying power developed in the mixture. At

certain stages of the reaction a constant relation exists between amount of acid liberated and the time of reaction. This does not apply at the beginning, nor at the end of the digestion when equilibrium is approached.

(10) The effect of continuous shaking is to inhibit the reaction. This may be due to a mechanical action of the enclosed air bubbles in diminishing the surface contact between zymolite and enzyme.

(11) The effect of rising temperature is to accelerate the reaction up to about 30°. Above that the reaction is inhibited, not by weakening of the enzyme at that temperature but by increasing the inhibitory effect of the oleic acid liberated. At 50° the enzyme is weakened, and at or before 60° it is destroyed. Within certain narrow limits of temperature the reaction follows the law of van't Hoff; in general it does not even approximate this law because of the other factors present which complicate the reaction.

ON A MODIFICATION OF LUNGE'S METHOD FOR THE QUANTITATIVE ESTIMATION OF UREA.

By CLARENCE QUINAN.

(*From the Hearst Laboratory of Pathology, University of California.*)

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Lunge,[1] in 1890, enriched the literature of analytical chemistry by a contribution of unusual interest dealing with the technique of gas analysis. We are indebted to him for a principle of great practical importance in this line of work, by means of which the errors incident to barometric calculations are virtually eliminated and thus a corresponding increase of accuracy insured.

Essentially, the innovation consists in the use, in connection with a gas measuring system, of a "reduction tube," or automatic barometer, thereby making it possible to reduce at will an arbitrary volume of any elementary gas to standard conditions of temperature and pressure.

Technical analysts were quick to recognize the advantages of a device so elegant both in theory and simplicity of application, and it is now in very general use in the larger laboratories. Though capable of rendering valuable service, it has remained, so far as I am aware, unknown to biological chemists.

Without entering into a discussion of the relative merits of the methods now in use for the quantitative estimation of urea, I desire to point out certain advantages of Lunge's principle as applied to the measurement of variable quantities of urea-nitrogen yielded in the well known hypobromite reaction. It seems well worth while to do this, because the hypobromite method with all its inaccuracies still finds a prominent place in current text-books, if not directly advocated by eminent authorities. Obviously, then, every opportunity should be taken advantage of to put it upon a better technical footing.

[1] *Zeitschr. f. angew. Chem.*, 1890, pp. 139–144.

I shall first consider briefly the original method, and then describe a modification which adds to its availability and effectiveness.

Lunge has stated his fundamental idea in the following generalization:[1]

Wenn man in einem "Reduktionsrohre" mittels eines "Niveaurohres" ein bekanntes Luftvolum unter solchen Druck versetzt, dass es dasselbe Volum einnimmt, welches es bei 0 und 760 mm. Barometerstand einnehmen wurde, und wenn mann genau denselben Druck auf ein anderes, unbekanntes Gasvolum ausübt, so wird auch das letztere den Raum einnehmen, welcher einer Temperatur von 0 und dem Luftdrucke 760 mm. entspricht. Dies wird erreicht, wenn erstens das "Niveaurohr" so hoch steht, dass das bekannte Volum im "Reduktionsrohr" auf die Normalien reduziert wird, wenn zweitens durch Anwendung eines T-Rohres derselbe Druck auch auf das Gasmessrohr wirkt, und drittens das Niveau des Quecksilbers in diesem genau ebenso hoch wie im "Reduktionsrohre" steht.

The apparatus commonly employed is figured in most standard works on volumetric analysis under the title of "gas-volumeter." It consists (Fig. 1) of two parts: a mercury-filled decomposition bulb, E, in which the gas is disengaged, and a measuring system of three tubes, also containing mercury. In this latter system, *B* is the reduction tube to which reference is made by the author, whilst in *A* the corrected gas reading is obtained. *C* and *F* are leveling tubes.

When filled with mercury the apparatus is quite heavy and should be mounted upon a stativ of solid construction provided with tube supports of such arrangement that *A* and *B* may be closely paralleled for comparison. The form devised by Lunge[2] is very convenient.

To establish *B* as a standard reduction tube, it is first necessary to calculate the volume which one hundred cubic centimeters of air would occupy at the temperature and pressure prevailing at the time of adjustment. The mercury in *A*, *B* and *C* being freely exposed to atmospheric pressure, the tubes are so arranged that in *B* the lower margin of the meniscus is at the calculated volume, The stopcock on *B* is then closed, and the apparatus is ready for use.

[1] *Chemische-technische Untersuchungsmethoden*, Berlin, i, p. 158, 1904.
[2] *Zeitschf. angrew. Chem.*, p. 175, 1892.

Simple as this procedure may appear, in practice it is by no means an easy matter to obtain an ideal result. Few laboratories it will be understood, possess temperature and pressure instruments of such extreme accuracy as to supply standard data for

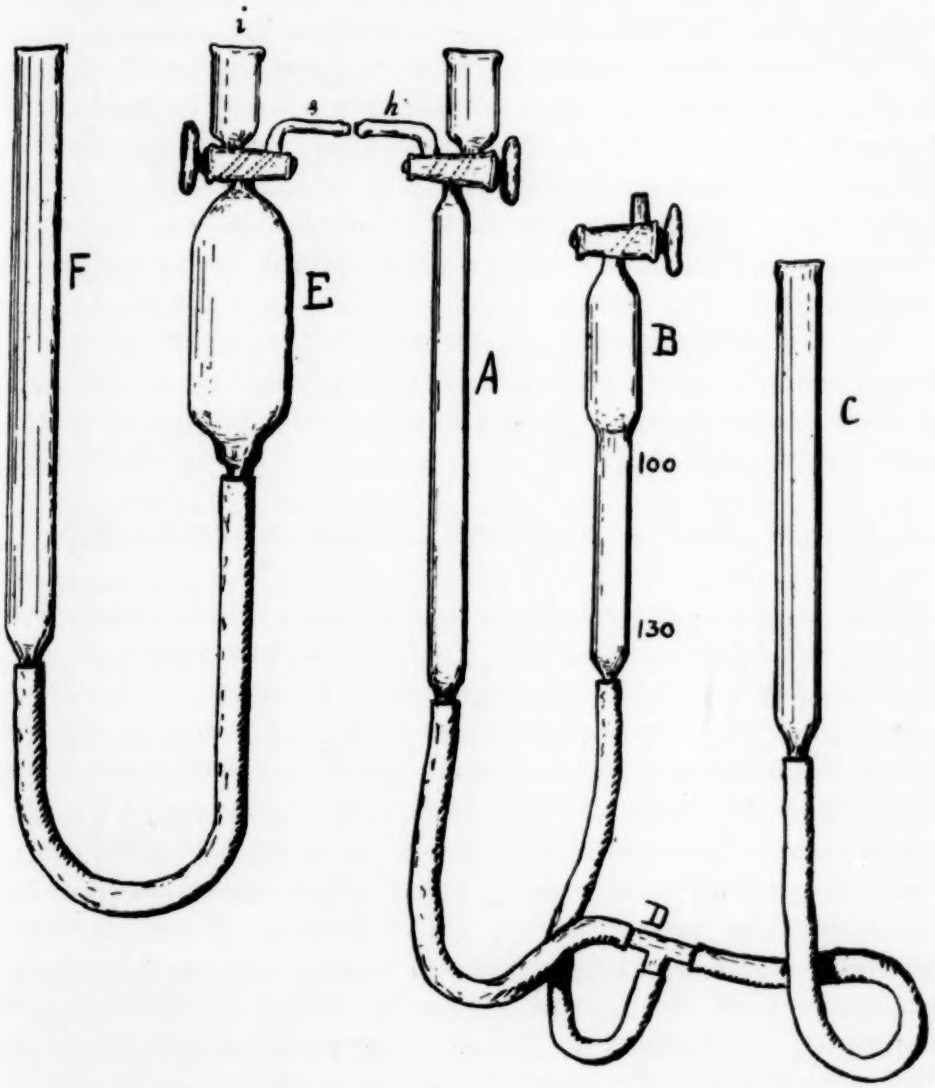

FIG. 1

calculation; then, too, a number of small corrections must be applied with constant possibilities of error, so it is hardly surprising that most chemists find it necessary to make an allowance ranging from 1.5 to 3 per cent for the error of standardization.

When the reduction tube has once been properly established, however, any volume of gas introduced into *A* may be read off at the normal value, by merely bringing *A* and *B* to the same level, the mercury in *B* having been forced to a volume of one hundred cubic centimeters by means of the leveling tube *C*.

Very likely the difficulties encountered in the preparation of this apparatus have tended to limit its usefulness to a certain degree, many who otherwise would have availed themselves of it having turned to other and less accurate appliances. Before taking up the technique of urea determinations, therefore, it will be necessary to consider a method by means of which the reduction tube may be standardized exactly without the aid of either thermometer or barometer.

The method depends upon the fact that potassium nitrate is decomposed in the presence of metallic mercury and an excess of sulphuric acid, all of its nitrogen being converted into nitric oxide according to the equation:

$$2KNO_3 + 4H_2SO_4 + 3Hg = K_2SO_4 + 3HgSO_4 + 2NO + 4\ H_2O.$$

The results of analysis show that we may without hesitation accept this equation as valid. If, then, a known weight of pure potassium nitrate be decomposed in this manner, and the liberated gas be transferred without loss to the measuring apparatus above described, it is obvious that a simple calculation will show the volume which the nitric oxide should occupy under normal conditions. This value being known, it is equally clear that, were it possible bv an arbitrary maneuver to make the measuring apparatus register the calculated volume, *A* and *B* being correctly leveled, and *B* indicating exactly one hundred cubic centimeters an ideal result would be achieved. Moreover, if successful, we should then possess a normal reduction tube, free from any element of correction.

As a matter of fact, this plan may be carried into execution, and the best possible evidence that it is a justifiable procedure is afforded by the analysis of other nitrates of different molecular weights. Assuming a fair degree of technical skill, uniformly concordant results are brought within easy reach by this means, the range of error in serial experiments upon pure nitrates not

exceeding 0.1 per cent. This, of course, would be out of the question were the method based upon an essentially vicious principle.

Technique of the modified method. The decomposition bulb *E*, both perforations in the two-way stopcock, and the nipple *g* are filled with mercury by means of the leveling tube *F*, which is then lowered to produce negative pressure in *E*, and clamped.

Since thirty cubic centimeters of nitric oxide at 0° and 760 mm. correspond very closely to 0.1357 gram of potassium nitrate, it is convenient to select that amount for decomposition. A salt of the greatest possible purity is absolutely necessary for obvious reasons, and it should be finely powdered and dried to constant weight before using. After weighing out, the salt, while still in the dry state, is brought into the thistle over *E*, where it is dissolved and rinsed into the chamber below, the object being to use very little water and thus prevent too great dilution of the reagents. Now draw in rapidly 15 to 20 cc. of concentrated sulphuric acid free from nitric acid—an acid negative to the ordinary tests, and which gives at most a very faint pink with brucine in substance, is permissible. Taking the bulb *E* out of its support, the contents are thoroughly mixed by a rotary or whirling movement which tends to prevent granulation of the mercury at the same time that it effects complete mixture. In a few minutes the reaction begins, a steady evolution of gas occurring until the decomposition is over. This requires from ten to fifteen minutes as a rule, though it it well to wait longer lest a trace of gas be lost.

The measuring apparatus having been prepared for use, *A* is filled with mercury by raising *C*, care being taken to fill the nipple tube *h*, at the same time. The stopcock on *B* must be closed while *A* is being filled, else the mercury properly required in the instrument will prove insufficient to fill *A*. With the nipples in the position shown in the figure, union is effected by means of thick-walled rubber tubing, the ends of the glass nipples being in apposition. The gas may now be transferred from *E* to *A* by simultaneous opening and closure of the corresponding stopcocks, previously controlling the pressure conditions by lowering *C* and raising *F*. Some little skill is required to effect the transfer of gas without permitting acid to enter *g*.

Nothing now remains but the adjustment of the reduction tube to the gas volume in *A*.

Inasmuch as the apparatus, when standardized, is to be used for measurements of urea-nitrogen only, and since this gas is not delivered to *A* in the dry state, the mercury both in *A* and *B* may be moistened with water before attempting the final manipulation to obviate an especial correction for the tension of water vapor. We may now admit an arbitrary volume of air to *B*, naturally, more than one hundred cubic centimeters; the stopcock is quickly turned, by means of the leveling tube the mercury in *B* is forced to the one hundred mark, and while there, *A* is brought as close as possible and the reading noted. This procedure must be repeated until, by good fortune, one captures the required volume of air in *B*. When finally successful, with the mercury in *B* at one hundred cubic centimeters and both tubes exactly leveled, *A* will read thirty cubic centimeters. If, after standing for some time to bring about equalization of temperature, the reading remains unchanged, we may accept it as correct and discharge the gas from *A*. Before making use of the apparatus in actual analysis, however, it is first necessary to apply a severe test. This is best done in the manner already mentioned, by analyzing a known weight of some other nitrate. In every instance it will be found that the instrument will record the theoretical volume corresponding to the weight of substance taken, provided that no errors were committed in the technique of preparation.

We now possess a normal measuring system, good for all time if the stopcock on the barometer remains airtight. For the recording of variable quantities of urea-nitrogen, the advantages of such a system are too obvious to require discussion.

Any desired volume or weight of urine or other urea-containing fluid may now be introduced into the decomposition bulb *E*, and there treated with an excess of freshly prepared sodium hypobromite solution, according to the usual practice. Owing to the stormy evolution of gas which attends the reaction, however, it is necessary to keep the leveling tube *F* very low, to insure negative pressure during the introduction of the hypobromite solution. After the absorption of the carbon dioxide has completed itself, the pure nitrogen is transferred to the reduction apparatus in the same manner as described for nitric oxide, and the true volume is read off.

Even the methylated purins are slowly attacked by sodium hypobromite, with separation of gas. In general, the sensitiveness of the purin bases to this reagent appears to be inversely proportional to their complexity of structure. In the small volume of urine ordinarily taken for analysis, however, uric acid is the only substance apt to be present in sufficient quantity to give rise to error and, in human urine at least, the inaccuracy due to this source must be very slight indeed.

THE RELATION OF DIFFERENT ACIDS TO THE PRECIPITATION OF CASEIN AND TO THE SOLUBILITY OF CHEESE CURDS IN SALT SOLUTION.[1]

By J. L. SAMMIS and E. B. HART.

(From the Departments of Dairy Husbandry and Agricultural Chemistry of the University of Wisconsin.)

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In a previous paper by one of us,[2] it was pointed out that the quantities of different acids required to produce visible curdling in milk are not chemically equivalent to each other, and that the proportion of any one acid required varies with the temperature. It was also pointed out that the addition of salts to milk produced quite as marked changes in the temperature of coagulation of milk as do acids.

The influence of barium chloride on the temperature of precipitation of lime water solutions of casein was shown, and the following brief reference was made to the action of acids on lime water casein solutions. "Van Slyke and Hart observed that when a lime water solution of casein is treated with an acid the liquid becomes intensely milky at room temperature before sufficient acid has been added to make the liquid neutral to phenolphthalein. No valid conclusion can be based on this phenomenon respecting the existence of neutral calcium casein in milk, or in the lime water casein solution, because the appearance of the milkiness in the liquid depends not only on the amount of acid added, but also on the temperature of the solution. Thus at 34° F., no milkiness whatever appears in the lime water casein solution until sufficient acid has been added to make the lime water casein solution quite strongly acid to phenolphthalein."

[1] Published by permission of the Director of the Wisconsin Experiment Station.

[2] J. L. Sammis: The Chemistry of Milk Curdling, Twenty-fourth Annual Report, Wisconsin Experiment Station.

It is clear that the definite percentage of lime in "calcium casein" calculated by Van Slyke and Hart from the neutralization of lime water casein solutions by acids holds only for the particular solutions, temperature and other conditions under which the experiment was performed, and therefore may not apply to milk.

The conclusion reached was that the coagulation of milk by acid does not alone depend upon the removal of lime from a definite compound, such as "calcium casein," in the manner suggested by Van Slyke and one of us[1] in an earlier paper, but is a reaction in which all of the milk constituents take part.

Since the theories referred to were based on studies of lime water casein solutions, while most of the experimental data presented in the paper quoted above were obtained from study of milk, it is the purpose now to record observations on coagulation of lime-water casein solutions with different acids at different temperatures, together with the solubility of the precipitates in salt solutions.

THE REACTIONS OF LIME WATER CASEIN SOLUTIONS WITH ACIDS.

The method of experiment was to add tenth-normal solutions of different acids to 10 cc. portions of a 1 per cent solution of casein in lime water, held at different temperatures, observing in each case how much acid was required (1) to remove the red color of the phenolphthalein indicator added, (2) to cause visible separation of a precipitate to begin, (3) to cause complete flocculation of the precipitate, (4) to give a precipitate completely and easily soluble in 5 per cent salt solution, and (5) to give a precipitate quite soluble in the salt solution. The precipitated casein was regarded as insoluble in salt solution when after treatment with salt solution and filtration, the filtrate did not become turbid on further addition of acid. The data tabulated below show that the quantity of acid required varied with the temperature and with the kind of acid in every case.

[1] Van Slyke and Hart: Casein and Paracasein in some of their Relations to Bases and Acids, *Amer. Chem. Journ.*, xxxiii, no. 5.

TABLE I.

Volumes of $\frac{N}{10}$ acids required for 10 cc. limewater casein solution.

KIND OF ACID.	EFFECT PRODUCED.	TEMPERATURE 5–10° C.	TEMPERATURE 18–20° C.	TEMP. 35° C.
		cc.	cc.	cc.
Lactic	1	3.25	3.20	3.00
	2	3.80	3.5	3.3
	3	4.10	3.8	3.6
	4	4.10	3.9	3.9
	5	4.30	4.20	4.0
Oxalic	1	2.65	2.60	2.50
	2	3.40	3.20	3.20
	3	3.50	3.50	3.40
	4	3.50	3.50	3.40
	5	3.7–3.9	3.70	3.60
Acetic	1	2.65	2.60	2.50
	2	3.95	3.50	2.9
	3	4.30	4.00	3.50
	4	4.50	4.20	4.20
	5	4.90	4.80	4.7
Phosphoric	1	1.80	1.80	1.70
	2	6.5	3.3	2.2
	3	6.5	4.0	2.9
	4	6.5	4.8–6.0	4.8–6.0
	5	7.0	6.7	6.7

Ten cc. of the lime water used in making the casein solution for the lactic acid experiment, was equal to 4.1 cc. $\frac{N}{10}$ acid, but the lime water used with the other acids equalled 3.50 cc.

AGE OF CASEIN SOLUTION, A FACTOR IN COAGULATION BY ACID.

The age of the lime water casein solution was found to have an important influence on the amount of acid required to react with it. A 1 per cent solution of casein was made, using lime water of which 10 cc. required 4.35 cc. $\frac{N}{10}$ hydrochloric acid to neutralize it. Part of this solution was used at once and part after twenty-four hours. Two experiments are tabulated below.

TABLE II.

Volumes of acid required for new and old casein solutions.

EFFECT PRODUCED.	AGE OF CASEIN SOLUTIONS.	
	Fresh.	24 hours old.
	cc.	cc.
A. (1)	3.40	2.80
(2)	3.90	3.70
(3)	4.00	3.90
(4)	4.35	....
(5)	....	4.35
B. (1)	2.50	2.25
(2)	3.65	3.30
(3)	3.70	3.50
(4)	4.30	....
(5)	....	4.30

CHANGE IN THE PROPERTIES OF CURD ON STANDING.

The precipitate formed in 10 cc. of a 1 per cent casein-lime water solution by adding 3.40 cc. of $\frac{N}{10}$ hydrochloric acid was found to be soluble in 5 per cent salt solution, if the liquid was decanted as soon as the precipitate had flocculated and the salt solution added at once and warmed. But if the liquid was left standing on the precipitate five minutes before decanting, the precipitate was found to be insoluble. On the other hand, when the liquid is decanted immediately and the drained curd is left alone for ten minutes, it is still perfectly soluble. These facts were observed repeatedly, and seemed to indicate that after the precipitation had occurred, the precipitate may undergo a further change on standing in contact with its mother liquor. Ten cubic centimeters of the lime water solution required 3.4 cc. $\frac{N}{10}$ acid for neutralization.

No evidence, however, of a progressive change in the liquid on standing before precipitation was seen. In these experiments 10 cc. of the lime water required 4.5 cc. $\frac{N}{10}$ acid for neutralization. Thus, 10 cc. portions of a 1 per cent casein-lime water solution turned white and opalescent at once when 3.8 or 4.00 cc. of acid were added, but no precipitation occurred on standing 24 hours, while 4.2 cc. of acid caused immediate precipitation.

In view of these facts, it may be desirable to describe one more experiment in which the casein solution was less than one hour old when used, and the salt solubility determinations were made after the precipitates had stood 5–10 minutes in contact with the mother liquor. Temperature of the experiment was 19-20° C. Ten cubic centimeters of lime water required 4.5 cc. $\frac{N}{10}$ acid.

TABLE III.

Different acids and their effect.

	$\frac{N}{10}$ H_2SO_4.	$\frac{N}{10}$ ACETIC.	$\frac{N}{10}$ OXALIC.	$\frac{N}{10}$ HCl.	$\frac{N}{10}$ H_3PO_4.	$\frac{N}{10}$ LACTIC.
	cc.	cc.	cc.	cc.	cc.	cc.
(1)	3.5	3.7	3.3	3.5	2.35	3.5
(2)	3.8	4.7	4.1	4.0	5.30	3.8
(3)	4.2	5.8	4.3	4.2	6.00	4.2
(4)	4.2	5.8	4.3	4.2	6.60	4.50
(5)	4.5	6.2	4.7	4.6	7.70	4.70

These results make it perfectly clear that unlike quantities of different acids are required to produce effects (1) to (5).

RELATION OF SALT SOLUBILITY OF CURDS TO PRECIPITATION OF MILK BY SALTS.

Since neutral salts in the absence of acids strongly influence the coagulation of milk or lime water-casein solutions, it appears very probable that the neutral salt formed on adding acid to lime water-casein solution has much to do with the precipitation of curds therefrom.

The solubility of cheese curds in 5 per cent common salt solution and other salt solutions is shown in the following table; the extractions were made with 75–100 cc. portions of the solvent and continued until 1000 cc. were collected.

TABLE IV.

Solubility of one day old cheddar cheese in 5 per cent solutions of various salts at 50–60° C.

	PER CENT OF THE TOTAL NITROGEN.
5 per cent NaCl	18.16
5 per cent KCl	36.00
5 per cent $MgCl_2$	61.78
5 per cent NH_4Cl	34.05
5 per cent $BaCl_2$	1.45
5 per cent $CaCl_2$	1.39

Solubility of cheese curds in various strengths of salts at 50–60°C., expressed in per cents of total N.

STRENGTH OF SOLUTION.	$CaCl_2$.	$BaCl_2$.	$MgCl_2$.	KCl.	NH_4Cl.	NaCl.
per cent.	*per cent.*	*per cent.*	*per cent.*	*per cent.*	*per cent.*	*per cent.*
.2	1.29	1.64	3.23	17.5	....	29.2
.5	1.61	1.80	3.65	28.1	33.8	41.3
1.0	2.14	1.80	5.68	40.8	44.4	53.6
2.5	2.59	2.10	57.82	52.2	64.8	64.2
5.0	2.42	2.15	72.74	60.2	67.9	61.5
10.0	2.14	1.85	71.51	66.0	57.8	56.7
15.0	1.45	1.92	61.85	65.6	36.2	19.8
5 per cent NaCl ...		60.6	58.1	67.1	65.0	61.5
Age of cheese in days .	4	6	8	12	14	18

The degree of solubility depends very much on the kind of salt and on the concentration in which it is employed. Potassium, sodium and ammonium chlorides are shown to have a strong solvent action on the cheese curd used, while barium and calcium chlorides have very little. Magnesium chloride is intermediate in the series, resembling the chlorides of the alkali metals, when used in the greater concentrations, and when used in low concentrations, resembling the chlorides of the alkaline earth metals. The addition of sodium, potassium, or ammonium chlorides to milk is known to markedly elevate its coagulation temperature while the chlorides of barium and calcium strongly depress the

coagulation temperature, when used at low concentration. In many experiments the curds formed by heat after adding various salts to milk, redissolved quickly when a little more of the same salt was added to the mixture.

INFLUENCE OF DIFFERENT ACIDS ON THE SALT SOLUBILITY OF CHEESE CURDS.

Since different acids in equivalent molecular proportions do not behave alike in curdling milk, it is likely that they will be found to influence the salt solubility of cheese curd, unequally.

To determine whether this is the case or not, 25 gram portions of fresh rennet curd were ground with sand, suspended in 100 cc. of water, treated with 8 cc. of normal acid and digested one hour at room temperature. The curds were then extracted with 500 cc. of water in small portions, and then by a 5 per cent sodium chloride solution to 1000 cc.

TABLE V.

KIND OF ACID ADDED.	PROPORTION OF THE TOTAL NITROGEN IN THE CHEESE.	
	Extracted by water.	Extracted by 5 per cent salt solution.
	per cent.	*per cent.*
(1) 8 cc. N H_2SO_4	3.62	37.1
(2) 8 " N HCl	19.38	42.4
(3) 8 " N H_3PO_4	3.88	35.5
(4) 8 " N $C_2H_4O_2$	4.59	73.6
(5) 8 " N $C_3H_6O_3$	4.46	64.2
(6) 8 " N $C_2H_2O_4$	42.01	49.9
(7) Blank	4.33	3.09

The phenomena of milk coagulation and salt solubility of cheese curds are affected by conditions of temperature, concentration and by the presence of many substances in solution, and with the discovery of new facts the older explanations of these phenomena become increasingly inadequate and incomplete.

AN ENDEAVOR TO ACCOUNT FOR THE TRANSFER OF PROTEID IN INANITION.

By ALBERT WOELFEL.

(From the Hull Physiological Laboratory, University of Chicago,)

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The work herewith reported consisted of a search for something about the lytic relations of different tissues which might be regarded as having significance with respect to the transfer of proteid from one organ to another, as it supposedly occurs in fasting animals.

As is well known, the proportional losses in weight of the different tissues during fasting do not proceed in parallel. The spleen, liver, and muscles, for example, undergo greater relative decreases in their substance than the heart and nervous tissues which preserve their weights for a long time almost undiminished. The latter organs are also the ones which manifest the least alteration in the discharge of their functions after a period of fasting. As the heart, especially, must be the seat of very intense metabolism for the performance of its considerable work, the inference has been that it draws proteid nutrition from the other organs, or as is commonly stated, it "feeds" on those organs which undergo a greater loss of substance during fasting.

In this connection some questions which suggest themselves and have not received consideration, I believe, are:

Might one not go to the length of questioning if the organs which waste least during fasting, depend at other times too for suitable nutrition on the wasting tissues; or if this is so during a state of inanition only, what then marks their turning to this dependence? If there is some influence constantly causing the wasting tissues to furnish some of their substance to the more persisting tissues, what is it? Or if this influence is exerted during fasting only, what conditions its operation? Or if it exists during fasting only, what gives rise to it?

It was thought that an answer to these questions, with reference to certain organs, might be found in a comparison of their autolytic properties and in a comparison of the extent of lysis in mixtures of them. This expectation was fostered by the evidence recently adduced that instead of the nutritive nitrogenous substances circulating in the form of albumens exclusively—as was formerly supposed—they might be transferred, partially at least, as fragments which are smaller than peptones represent.[1] Moreover Embden and Knoop[2] found albumoses sometimes in the blood-serum of fasting animals. The involvement of a lytic process in the supplying of the necessary nitrogenous nutrition to the more persisting organs during fasting is also suggested by the results of Claypon and Schryver,[3] who found that there was more extensive autolysis in certain tissues, especially the liver, taken from previously starved animals than in the same tissues from well-fed animals.

On the other hand, there were, indeed, misgivings as to the significance of whatever results might be obtained, because as yet autolysis has not really been shown to play any rôle in physiological processes, even though Langstein and Neubauer and Ferroni[4] found that the yield of non-coagulable nitrogenous substances from autolysis of puerperal uteri was greater than that from normal ones. In fact, by some the phenomenon is looked upon, it seems, as a mere biological curiosity because autolysis is generally most favored by an acid reaction[5] and is almost nil in an alkaline reaction; and because blood serum seems to contain antienzymes which inhibit the autolysis of most tissues[6] and because during autolysis substances are formed character-

[1] Leathes: *Problems in Animal Metabolism*, 1906, pp. 124–141.

[2] Embden und Knoop: *Beitr. z. chem. Physiol. u Pathol.*, iii, p. 120, 1902.

[3] Claypon and Schryver: *Journ. of Physiol.*, xxxi, p. 169.

[4] Langstein u. Neubauer: *Münch. med. Wochenschr.*, xlix, p. 30, 1902; Ferroni: cited from *Biochem. Centralbl.*, v, no. 2198.

[5] Mendel and Leavenworth: *Amer. Journ. of Physiol.*, vol. xxi, p. 69, 1908; Hedin: *Festschrift für O. Hammarsten*, 1906; v'Drjewezki: *Biochem. Zeitschr.*, i, p. 229; Weiner: *Centralbl. f. Physiol.*, xix, p. 349; Dakin: *Journ. of Physiol.*, xxx, p. 84; Leathes: *Journ. of Physiol.*, xxviii, p. 360.

[6] Longcope: *Journ. of Med. Res.*, xiii, p. 45; Opie, *Proc. of the Soc. for Exp. Biol. and Med.*, New York, May, 1905; Baer und Loeb: *Arch. f. exp. Path. u. Pharm.*, liii, p. 1; Hedin: *Journ. of Physiol.* vol. xxx.

istically, which are not known to have physiological uses. The observations reported on two at least of these points are so much at variance,[1] however, that it is left an open question yet as to whether or not processes represented by autolysis or by heterolysis *in vitro* are involved in physiological processes. Besides, Leathes,[2] on the basis of evidence furnished by Ehrlich and Lazarus[3] that the reaction in and around the nuclei of white corpuscles is acid, questions if the generally observed unfavorable influence of alkaline medium on the action of autolysis is any obstacle to ascribing to them a rôle in intracellular metabolism since they might operate in the neighborhood of the nucleus.

On the other hand, the operation of autolysins as well as heterolysins in some pathological conditions seems certain. According to Wells[4] a process quite similar to autolysis is involved in the removal of infarcts. The influencing of lysis of one tissue by another, as is illustrated by the part which leucocytes take in removing infarcts (Wells), and in causing the resolution of lobar pneumonia is generally recognized.[5]

Might it not be possible that other cells than leucocytes furnish substances which have lytic influences on other, though living, tissues and might it not be possible that in starvation this effect may be exerted through the circulating media between the tissues which must be maintained and those which diminish in substance? And if so, is it by virtue of a digestive power which the persisting tissues may always have on the wasting tissues, but which is exercised only during fasting of the animal when the blood-plasma or the wasting tissues themselves may become deficient in antienzymes? Or might it be that fasting furnishes a condition which favors the autolysis of those tissues which waste?

It was thought that, if any of these things are true, it might be manifested by a comparison of the autolysis of tissues from fasting and from normal animals; or by comparing the lysis of wasting tissues from fasting and from normal animals mixed with

[1] Preti: *Zeitschr. f. physiol. Chem.*, lii; Baer: *Arch, f. exp. Path. u. Pharm.*, lvi.

[2] Leathes: *Journ. of Physiol.*, xxviii.

[3] Ehrlich und Lazarus, in Nothnagel's *Specielle Path. u. Ther.*, viii, p. 31.

[4] Wells: *Journ. of Med. Res.*, xv, p. 149.

[5] Simon: *Deutsch. Arch. f. klin. Med.*, lxx; Müller: *Congress für innere Medizin*, 1902; Silvestrini: *Biochem. Centralbl.*, i, no. 1599.

blood serum from fasting and from normal animals; or by observing the heterolysis in mixtures of persisting tissues from normal and from fasting animals with wasting tissues from normal and from fasting animals.

The tests made in this direction were confined to muscle, brain, heart, and serum. The results obtained were quite indifferent as to their significance. So, in keeping with the value of such results, this report, having discussed the motive, will set forth further only the salient points about the methods and results of some of rather numerous tests.

The tissues used were taken from animals which, after anæsthetization with ether, had been bled and their bodies made as blood-free as possible by perfusion with a solution which might be called plasma-salt solution. The blood of the animals was also carefully collected, defibrinated and then centrifuged to obtain as much clear serum as possible. The blood-free tissues were minced very fine.

Measured volumes of blood serum and weighed amounts of minced tissue were allowed to autolyze; mixtures of tissues, and mixtures of tissue with serum were allowed to digest. In all cases the digesting substances were mixed with an antiseptic fluid, toluol or chloroform, and with plasma-salt solution, the whole volume of the fluid being uniform in a given series, that is, where blood-serum was a part of the digesting mixture, the volume of plasma-salt solution used was correspondingly diminished.

This plasma-salt solution consisted of a solution of *all* known inorganic constituents (except ammonia) of blood plasma in about the same amount per volume as they occur (Hammarsten) in blood-plasma of cats. This solution was made by mixing in the following order: solutions of the proper amounts of sodium sulphate, potassium chloride, magnesium sulphate, sodium chloride, sodium bicarbonate, calcium chloride and disodium phosphate. Such a solution was designed to furnish a medium which would represent the blood-plasma qualitatively, as far as its inorganic constituents are concerned, because it is said to be not a matter of indifference to such enzymes[1] as here concern us, what media they are in—although in such a solution the relation of the ions

[1] Launoy: *Compt. rend. soc. biol.*, lxii, pp. 487 and 1175; Galdi, *Biochem. Centralbl.*, iii, no. 2088.

to each other would supposably not be the same as in real plasma. It was presumed also that the albumens and enzymes of the tissues would be dissolved and hence interact better in such a salt solution than otherwise. The plasma salt solution was alkaline, notwithstanding, as has already been noted, that an acid reaction has usually been found to be most favorable to autolysis *in vitro*. In these tests, however, it was desired to have an alkaline medium like that of the body fluids, so that, if striking differences of the kind sought showed themselves, one would be more warranted in applying them to the questions under consideration than if the reaction of the digesting mixtures was so far removed from that of the natural tissues in general. Furthermore the plasma-salt solution was of *known* alkalinity. When, therefore, it was desired to neutralize the digesting mixture before heating to remove coagulable substances, an exactly requisite amount of acid solution could be used instead of adding dilute acid haphazard as is usually done; the uniformity which is thus secured is of great importance in the coagulation procedure.

The flasks containing the mixtures were properly corked and put into an incubator at about 37° for periods ranging from 10 or 14 days to 7 weeks. The flasks were well shaken every day during the digestion.

After the digestions had proceeded as far as desired, the relative amounts of nitrogen in the non-coagulable substances of the fluid were determined by the following procedure: The fluid of each flask was poured through a strainer into a half-liter flask, the solid residue remaining was shaken up or washed several times with plasma-salt solution and the latter poured into the measuring flask until it was filled to the mark. The filled measuring flasks were allowed to stand over night to allow their contents to sediment. Most of the supernatant fluid was then carefully siphoned off from each flask and filtered and refiltered until the filtrate was quite clear; 250 cc. of each filtrate was measured into a 300 cc. flask and to each was added 50 cc. of dilute acetic acid and mono-sodium-phosphate solution of such strength that it could exactly neutralize 250 cc. of the plasma-salt solution used. The flasks containing the neutralized fluids were plugged with cotton and immersed in boiling water for one-half hour. The contents of each of these flasks, after cooling and filling to the

mark with distilled water, were filtered and then 250 cc. of each filtrate measured into a Kjeldahl flask and digested with sulphuric acid. The rest of the procedure was after the usual Kjeldahl nitrogen determination method.

As a matter of fact, however, in all the series where normal tissues alone were used and in one of the series where tissues from both normal and fasting animals were used, determinations of the total nitrogen, as well as determinations of nitrogen in the non-coagulable substances, in the fluid portions of the digestings were made also. As such determinations show nothing noteworthy, they are omitted from the tabulations of results.

As has just been intimated, a number of series were done on normal tissues alone. These were done preliminarily to make certain that the results of lyses in mixtures of tissues of normal animals, muscle, heart, brain and serum were fairly constant as between different animals and to test the feasibility of the method devised for the object in view. Such determinations also serve to test one of the questions proposed, viz: do the persisting tissues of normal animals possess something which increases lysis when they are mixed with wasting tissues? The experiments tabulated below serve this latter purpose also, so the results of these preliminary tests on tissues of normal animals alone need not be given.

Of course, comparisons of boiled tissues with unboiled tissues were made as usual in order to feel assured that the greater part of the non-coagulable nitrogen found in the fluids after digestion was from the products of autolysis. This proved to be the case with muscle, brain and heart, but usually to a less extent with blood-serum.

Since it is said that some ammonia is formed during autolysis, it was thought possible that in tests such as these with an alkaline medium there might be a loss from this source. Two series of comparisons, one with cat's, the other with goat's sera, of total nitrogen before autolysis and after autolysis were made. Since of the two series, only that on cat's sera showed considerable autolysis, it alone can be taken to show that a loss by ammonia formation in autolysis is negligible. This is shown in table I.

The results of two series of tests, performed in accordance with

the above described procedure, both on tissues from normal animals and from fasting animals are shown in tables II and III. Table II presents the results of lyses of cat's tissues, using toluol as an antiseptic, table III of goat's, using choloroform as an antiseptic. Under (*a*) are given the amounts of non-coagulable nitrogen derived from autolyzing tissues; under (*b*) are given the amounts of non-coagulable nitrogen obtained from digesting mixtures and accompanying these figures in each case is the figure obtained by computing according to (*a*) the sum of the non-coagulable nitrogen which would be obtained from the tissues used in the mixture had they been allowed to autolyze separately. In the last columns are given the numerical relations, $\frac{M}{S}$, between the amounts of non-coagulable nitrogen, obtained from the mixtures and that computed for separate autolysis.

The results given in these two tables, while they present irregularities, are on the whole as concordant as could be expected where such small amounts of tissues—without sacrificing many animals—had to be used, and the lysis was so slight owing to the alkaline medium.

It will be noted that, under the conditions, the blood-free tissues tested from fasting animals show no marked difference in their autolytic properties from the tissues of normal animals; hence there is no evidence of increase of autolysins or of diminution of antienzymes in them. For the mixtures of fixed tissues, $\frac{M}{S}$ usually approximates 1.0 and hence the persisting tissues both from normal and from fasting animals cannot be said to increase lysis when mixed with the wasting tissues. The most marked digressions from $\frac{M}{S} = 1.0$ are in the cases of blood-sera mixed with muscle of fasting cat; they would argue against the supposition of a lysin for wasting tissues circulating in the blood of fasting animals or a deficiency of antienzymes in it, especially as the digressions are in the direction downward from unity; if they mean anything, they are either manifestations of the antienzymes of blood-serum or are due to diminution of digestible substance in starved muscle though the autolysis of starved muscle controverts this; the blood-sera of goats mixed with starved muscle showed some digression in the opposite direction, however.

The results of determinations of the non-coagulable nitrogen

in toto, resulting from the various tests, must then be regarded as indifferent as to their significance. While they do not answer any of the questions set up, neither are they negations of any of them. Some of the suspected factors might exist but not be operable under the conditions of the tests, dead tissues impregnated by antiseptics. And it still remains a question—though probably far-fetched—if, instead of determining the non-coagulable nitrogen *in toto*, a search for qualitative differences resulting from the lysis of the different tissues or mixtures of them might not reveal something of significance in this connection.

TABLE I.

	50 cc. of	Total N.	Non-coagulable N.	Non-coag. N / Total N.
		gram.	*gram.*	
1	Normal cat's serum	0.2726	0.0279	0.1020
2	Normal cat's serum	0.2709	0.0253	0.0934
3	Normal cat's serum autolyzed	0.2705	0.0453	0.1670
4	Normal cat's serum autolyzed	0.2726	0.0455	0.1660
5	Starved cat's serum	0.2420*	0.0220	0.0909
6	Starved cat's serum	0.2429	0.0228	0.0940
7	Starved cat's serum autolyzed	0.2402	0.0459	0.1910
8	Starved cat's serum autolyzed	0.2402	0.0455	0.1890

* The fact that the total N of starved cat's blood-serum is here less than that of normal cat's serum is of no significance because smaller quantities of both sera than were needed for the above tests were obtainable, so both were diluted with plasma-salt solution to "stretch" them to the required total quantities. Dilution of the sera in the experiments of Tables II and III had to be resorted to, also.

TABLE II a.

	AUTOLYSIS OF	NON-COAGULABLE N.
		gram.
1	9.73 grams normal cat's muscle	0.0969
2	10.00 grams normal cat's muscle	0.1011
3	9.89 grams starved cat's muscle	0.0938
4	10.26 grams starved cat's muscle	0.1001
5	3.24 grams normal cat's heart	0.0164
6	2.65 grams normal cat's heart	0.0143
7	3.73 grams starved cat's heart	0.0166
8	3.64 grams starved cat's heart	0.0182
9	25 cc. normal cat's serum	0.0042
10	25 cc. normal cat's serum	0.0042
11	25 cc. starved cat's serum	0.0049
12	25 cc. starved cat's serum	0.0052

TABLE III a.

	AUTOLYSIS OF	NON-COAGULABLE N.
		gram.
1	20.95 grams normal goat's muscle	0.074
2	21.0 grams normal goat's muscle	0.0756
3	20.8 grams starved goat's muscle	0.0746
4	21.5 grams starved goat's muscle	0.0786
5	9.05 grams normal goat's heart	0.0282
6	8.0 grams normal goat's heart	0.0263
7	9.25 grams starved goat's heart	0.0262
8	8.7 grams starved goat's heart	0.0245
9	13.9 grams normal goat's brain	0.0474
10	11.28 grams normal goat's brain	0.0401
11	10.32 grams starved goat's brain	0.0366
12	10.1 grams starved goat's brain	0.0341
13	50 cc. normal goat's serum	0.0327
14	50 cc. normal goat's serum	0.0332
15	50 cc. starved goat's serum	0.0294
16	50 cc. starved goat's serum	0.0300

TABLE II b.

	LYSIS OF	NON-COAGULABLE N.	$\frac{M}{S}$
		gram.	
13	10.24 grams normal muscle and 2.03 grams normal heart, mixed	0.1183	1.042
	10.24 " " " " 2.03 " " " separately	0.1134	
14	10.34 grams normal muscle and 2.12 grams normal heart, mixed	0.1151	1.002
	10.34 " " " " 2.12 " " " separately	0.1149	
15	10.21 grams normal muscle and 3.11 grams starved heart, mixed	0.1214	1.04
	10.21 " " " " 3.11 " " " separately	0.1167	
16	10.2 grams normal muscle and 2.21 grams starved heart, mixed	0.1151	1.023
	10.2 " " " " 2.21 " " " separately	0.1125	
17	10.31 grams starved muscle and 2.11 grams normal heart, mixed	0.1029	.933
	10.31 " " " " 2.11 " " " separately	0.1101	
18	10.03 grams starved muscle and 2.04 grams normal heart, mixed	0.1046	.976
	10.03 " " " " 2.04 " " " separately	0.1071	
19	10.57 grams starved muscle and 2.2 grams starved heart, mixed	0.112	1.002
	10.57 " " " " 2.2 " " " separately	0.1117	
20	10.22 grams starved muscle and 2.18 grams starved heart, mixed	0.1095	1.011
	10.22 " " " " 2.18 " " " separately	0.1082	
21	10.0 grams normal muscle and 25 cc. normal serum, mixed	0.1046	1.000
	10.0 " " " " 25 cc. " " separately	0.1045	
22	10.43 grams normal muscle and 25 cc. normal serum, mixed	0.1086	0.997
	10.43 " " " " 25 cc. " " separately	0.1089	
23	9.75 grams normal muscle and 25 cc. starved serum, mixed	0.1004	0.975
	9.75 " " " " 25 cc. " " separately	0.1029	
24	10.23 grams normal muscle and 25 cc. starved serum, mixed	0.1039	0.964
	10.23 " " " " 25 cc. " " separately	0.1077	

25	10.18 grams starved muscle and 25 cc. normal serum, mixed	0.0871	0.853
	10.18 " " " " 25 cc. " " separately	0.1021	
26	10.53 grams starved muscle and 25 cc. normal serum, mixed	0.0917	0.869
	10.53 " " " " 25 cc. " " separately	0.1054	
27	10.27 grams starved muscle and 25 cc. starved serum, mixed	0.0882	0.849
	10.27 " " " " 25 cc. " " separately	0.1038	
28	10.09 grams starved muscle and 25 cc. starved serum, mixed	0.0854	0.836
	10.09 " " " " 25 cc. " " separately	0.1021	

TABLE III b

	LYSIS OF	NON-COAGULABLE N.	M/S
		gram.	
17	21.1 grams normal muscle and 8.3 grams normal heart, mixed	0.0969	0.956
	21.1 " " " " 8.3 " " " " separately	0.1013	
18	20.0 grams normal muscle and 9.8 grams normal heart, mixed	0.0974	0.954
	20.0 " " " " 9.8 " " " " separately	0.1021	
19	21.0 grams normal muscle and 8.27 grams starved heart, mixed	0.0908	0.925
	21.0 " " " " 8.27 " " " " separately	0.0981	
20	23.2 grams normal muscle and 9.75 grams starved heart, mixed	0.0987	0.896
	23.2 " " " " 9.75 " " " " separately	0.1102	
21	20.83 grams starved muscle and 10.17 grams normal heart, mixed	0.1060	0.987
	20.83 " " " " 10.17 " " " " separately	0.1074	
22	22.68 grams starved muscle and 9.67 grams normal heart, mixed	0.1021	0.902
	22.68 " " " " 9.67 " " " " separately	0.1131	

TABLE III b Continued.

	LYSIS OF	NON-COAGULABLE N.	$\frac{M}{S}$
		gram	
23	21.63 grams starved muscle and 9.47 grams starved heart, mixed	0.1028	0.979
	21.63 " " " " 9.47 " " " separately	0.1050	
24	22.36 grams starved muscle and 9.09 grams starved heart, mixed	0.1038	0.974
	22.36 " " " " 9.09 " " " separately	0.1065	
25	20.2 grams normal muscle and 11.25 grams normal brain, mixed	0.1088	0.978
	20.2 " " " " 11.25 " " " separately	0.1112	
26	20.57 grams normal muscle and 10.98 grams normal brain, mixed	0.1128	1.011
	20.57 " " " " 10.98 " " " separately	0.1116	
27	20.51 grams normal muscle and 11.42 grams starved brain, mixed	0.1108	0.983
	20.51 " " " " 11.42 " " " separately	0.1126	
28	20.14 grams normal muscle and 11.11 grams starved brain, mixed	0.1058	0.959
	20.14 " " " " 11.11 " " " separately	0.1102	
29	21.55 grams starved muscle and 11.3 grams normal brain, mixed	0.1165	0.992
	21.55 " " " " 11.3 " " " separately	0.1174	
30	21.71 grams starved muscle and 11.05 grams normal brain, mixed	0.1186	1.012
	21.71 " " " " 11.05 " " " separately	0.1171	
31	21.65 grams starved muscle and 11.53 grams starved brain, mixed	0.1165	0.985
	21.65 " " " " 11.53 " " " separately	0.1183	
32	26.57 grams starved muscle and 12.23 grams starved brain, mixed	0.1322	0.954
	26.57 " " " " 12.23 " " " separately	0.1385	
33	21.29 grams normal muscle and 50 cc. normal serum, mixed	0.1085	0.996
	21.29 " " " " 50 cc. " " separately	0.1089	
34	20.4 grams normal muscle and 50 cc. normal serum, mixed	0.1058	1.000
	20.4 " " " " 50 cc. " " separately	0.1057	

35	20.96 grams normal muscle and 50 cc. starved serum, mixed	0.1088	1.041
	20.96 " " " " 50 cc. " " separately	0.1044	
36	20.35 grams normal muscle and 50 cc. starved serum, mixed	0.1068	1.044
	20.35 " " " " 50 cc. " " separately	0.1023	
37	19.95 grams starved muscle and 50 cc. normal serum, mixed	0.1130	1.074
	19.95 " " " " 50 cc. " " separately	0.1052	
38	20.35 grams starved muscle and 50 cc. normal serum, mixed	0.1132	1.061
	20.35 " " " " 50 cc. " " separately	0.1067	
39	20.03 grams starved muscle and 50 cc. starved serum, mixed	0.1095	1.070
	20.03 " " " " 50 cc. " " separately	0.1022	
40	20.58 grams starved muscle and 50 cc. starved serum, mixed	0.1113	1.063
	20.58 " " " " 50 cc. " " separately	0.1047	

THE MODE OF OXIDATION IN THE ANIMAL ORGANISM OF PHENYL DERIVATIVES OF FATTY ACIDS. PART IV.

FURTHER STUDIES ON THE FATE OF PHENYLPROPIONIC ACID AND SOME OF ITS DERIVATIVES.

By H. D. DAKIN.

(*From the Laboratory of Dr. C. A. Herter, New York.*)

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In previous communications[1] it was shown that phenylpropionic acid, at least in part, underwent catabolism in the animal organism in accordance with the following scheme:

$C_6H_5.CH_2.CH_2.COOH$
(Phenylpropionic acid)
↓
$C_6H_5.CH(OH).CH_2.COOH$
(phenyl-β-oxypropionic acid)
↓
$C_6H_5.CO.CH_2.COOH$
(benzoylacetic acid)
↓
$C_6H_5.CO.CH_3$
(acetophenone)
↓
$C_6H_5.COOH$
(benzoic acid)
↓
$C_6H_5.CO.NH.CH_2.COOH$
(hippuric acid)

At the time of writing the first communication it was not found possible to obtain direct evidence of the production of benzoylacetic acid, but its formation as an intermediate stage in the oxidation was inferred from the detection of acetophenone into which it readily passes through loss of carbon dioxide.

[1] *Beitr. z. chem. Physiol. u. Pathol.*, xi, p. 404, 1908; this *Journal*, iv, p. 419, 1908; *ibid.*, v, p. 303, 1908.

Qualitative evidence of the excretion of benzoylacetic acid in the urines of animals which have received injections of considerable quantities of the sodium salts of both phenylpropionic acid and phenyl-β-oxypropionic acid has now been obtained. The excretion of the other intermediary substances represented in the foregoing scheme was satisfactorily demonstrated. Incidentally two new confirmatory tests for the detection of phenyl-β-oxypropionic acid have been successfully applied. Further investigation has shown that in addition to the foregoing substances, under certain circumstances, another derivative of phenylpropionic acid is excreted in the urines of animals which have received injections of sodium phenylpropionate. This body, which in many respects resembles hippuric acid, has been identified as cinnamoylglycocoll, $C_6H_5.CH:CH.CONH.CH_2.COOH$, and its synthesis was described in a recent number of this *Journal*.[1] The mechanism of formation of this substance is by no means clear. The most obvious explanation would be that it is formed by the coupling of glycocoll with cinnamic acid derived from phenyl-β-oxypropionic acid through loss of the elements of water.

$$C_6H_5.CHOH.CH_2.COOH \rightarrow C_6H_5.CH:CH.COOH$$
$$\rightarrow C_6H_5.CH:CH.CO.NH.CH_2.COOH$$

It is not certain if this is really the correct view, for in this case one would expect that cinnamoylglycocoll would be readily obtained on administering cinnamic acid to an animal. Actual experiment showed that cinnamic acid was very readily oxidized in the body and, although qualitative evidence of the possible formation of cinnamoylglycocoll was obtained in the case of both cats and dogs, the amount of substance thus found was insignificant. Incidentally, some interesting observations were made on the mode of combustion of cinnamic acid in the body which will be referred to later.

An alternative hypothesis would be that the cinnamoylglycocoll was derived from phenyl-β-oxypropionylglycocoll through the removal of a molecule of water—a change which is readily brought about *in vitro* by means of hydrochloric acid.[2]

$$C_6H_5.CHOH.CH_2.CO.NH.CH_2.COOH$$
$$= C_6H_5.CH:CH.CO.NH.CH_2.COOH + H_2O$$

[1] This *Journal*, v, p. 303, 1908.
[2] This *Journal*, v, p. 309, 1908.

Phenyl-β-oxypropionylglycocoll was synthesized by the method given in the communication previously referred to and was administered in the form of its sodium salt to both cats and dogs. In every case almost all of the substance was excreted unchanged and indications of the excretion of only very small amounts of cinnamoylglycocoll could be obtained. That cinnamoylglycocoll originates from phenyl-β-oxypropionylglycocoll therefore appears improbable. Somewhat more decided indications of the production of cinnamoylglycocoll were obtained when phenyl-β-oxypropionic acid, or rather its sodium salt, was administered, but here again the amount was not large.

The largest yields of cinnamoylglycocoll have been obtained from urines secreted after administration of the salts of phenylpropionic acid, phenylpropionylglycocoll and phenylvaleric acid.

At the same time it must be mentioned that the conditions for the formation of this substance are not yet clearly defined. From the urines of two cats which received identical doses of phenylpropionic acid (1.0 gram per kilo), there was obtained in one case no less than 0.45 gram of pure crystalline cinnamoylglycocoll (m. p., 192–193°) in addition to hippuric acid and other products, while in the other case only faint qualitative indications of cinnamoylglycocoll could be obtained, although much hippuric acid was present.

It is possible that cinnamoylglycocoll may be derived from phenylpropionic acid by first coupling with glycocoll and then undergoing oxidation with formation of cinnamoylglycocoll without going through the stage of phenyl-β-oxypropionylglycocoll:

$$C_6H_5.CH_2.CH_2.COOH \rightarrow C_6H_5.CH_2.CH_2.CO.NH.CH_2.COOH$$
$$\rightarrow C_6H_5.CH:CH.CO.NH.CH_2.COOH$$

This hypothesis would accord well with the facts, but cannot be considered proven especially as similar reactions, so far as I am aware, have not been hitherto observed. All that the facts really warrant is the statement that when phenylpropionic acid and certain substances related to it are administered to animals, the phenylpropionyl grouping may be converted by oxidation into a cinnamoyl-grouping which, combined with glycocoll, is excreted in the urine as cinnamoylglycocoll. Whether the gly-

cocoll addition is a primary or secondary reaction is not certain, but the balance of evidence is in favor of the latter view.[1]

Cinnamoylglycocoll itself on administration to cats was found to be excreted practically unchanged, so that it is improbable that cinnamoylglycocoll represents a normal stage in the catabolism of phenylpropionic acid. Its occurrence in the urine may be taken as an indication of an effort on the part of the organism to protect itself against the toxic properties of some of the oxidation products of phenylpropionic acid.

The mode of oxidation of cinnamic acid. The identification of cinnamoylglycocoll as a product of the catabolism of phenylpropionic acid made it necessary to investigate the fate in the organism of cinnamic acid itself. The acid was administered subcutaneously in the form of its ammonium salts (the sodium salt being too insoluble) to both cats and dogs in doses varying from 0.25 gram to 0.45 gram per kilo body weight. In every case a large amount of hippuric acid was excreted in agreement with the old observation of Erdmann and Marchand,[2] together with a small amount of a substance which appeared to be cinnamoylglycocoll, but which was not present in sufficient quantity to permit of isolation. *In addition acetophenone and phenyl-β-oxypropionic acid were detected.* This result appears to be of great interest, as it furnishes an example of the easy interconversion of an unsaturated acid into the corresponding β-oxy-acid. The only similar case with which I am acquainted is that of Friedmann[3] who found that crotonic acid when perfused through

[1] Reference may be made to a single experiment in which a two per cent solution of sodium cinnamate (10.0 grams) and glycocoll (5.0 grams) was slowly introduced into the femoral vein of a dog (10 kilos). The dog died after about two and one-half hours. The urine excreted during the experiment contained no appreciable amount of cinnamoylglycocoll. The blood, liver, heart, lungs, kidneys and spleen were removed and after mincing the mass was extracted with boiling water. The aqueous filtrate was concentrated but yielded on examination for cinnamoylglycocoll in the usual way only a small quantity of cinnamic acid (0.23 gram). The small yield of cinnamic acid makes it appear probable that the bulk of the acid entered into combination with the proteins or other substances in the body. The somewhat similar behavior of salicylic acid, recently investigated by Jacoby (*Biochem. Zeitschr.*, ix, p. 522) may be referred to in this connection.

[2] Liebig's *Annalen*, xliv, p. 344.

[3] Hofmeister's *Beiträge*, xi, p. 371.

a surviving liver yielded acetone. Friedmann assumes that β-oxybutyric acid was first formed, but did not isolate the substance. The oxybutyric acid then yields acetone through acetoacetic acid in the usual way.

In the case of cinnamic acid it was possible to obtain good evidence of the presence of laevorotatory phenyl-β-oxypropionic acid (p. 214). The acetophenone also detected in the urine doubtless originates, in part at any rate, from the further oxidation of the phenyl-β-oxypropionic acid, through benzoylacetic acid. The question whether any benzoylacetic acid or acetophenone is formed by direct oxidation of cinnamic acid without going through the stage of phenyl-β-oxypropionic acid cannot at present be answered.

Since phenyl-β-oxypropionic acid appears at least to some extent to give cinnamic acid—or rather its derivative, cinnamoylglycocoll, in the body, it would seem very probable that the conversion of the β-oxy-acid into the unsaturated acid and vice versa is a reversible process. This result is of considerable interest in connection with the relations and mode of origin of unsaturated acids, and will be referred to in a later paper in which some general considerations of fatty acid metabolism will be touched upon. The mode of oxidation of cinnamic acid in the body may be represented as follows:

$$C_6H_5.CH{:}CH.COOH \rightleftarrows C_6H_5.CH(OH).CH_2.COOH$$

$$\cdots\cdots ? \cdots\cdots \quad \swarrow$$

$$C_6H_5.CO.CH_2.COOH$$

$$\downarrow$$

$$C_6H_5..CO.CH_3$$

$$\downarrow$$

$$C_6H_5.COOH$$

$$\downarrow$$

$$C_6H_5.CO.NH.CH_2.COOH$$

The direct conversion of an unsaturated acid into the corresponding oxy-acid outside the body is usually difficult and not very many cases are known. As an example of this the conversion of fumaric acid into malic acid when heated for fifty-six hours with caustic soda solution may be cited. The reverse change, namely, the conversion of the β-oxy-acids into unsaturated acids, is of course much more common.

EXPERIMENTAL PART.

The fate of phenylpropionic acid. In previous communications it was shown that phenyl-β-oxypropionic acid and acetophenone were intermediary products in the conversion in the body of phenylpropionic acid into hippuric acid. The additional data here presented concern the isolation of cinnamoylglycocoll and the detection of benzoylacetic acid. In addition two qualitative tests for the detection of phenyl-β-oxypropionic acid are described.

Cinnamoylglycocoll was successfully isolated as follows: 4.0 grams of phenylpropionic acid[1] was neutralized exactly with caustic soda and the sterile solution injected subcutaneously into a cat weighing 5.0 kilos. The cat vomited some recently taken food and was quieter than usual for several hours, but drank milk and water freely. The animal gradually sank into a comatose condition and died about sixty hours after the injection had been given. There were no significant local changes at the site of injection. A small part of the urine was distilled and the acetophenone in the distillate was estimated in the usual way by the iodine method. 0.12 gram of acetophenone was found, calculated for the whole quantity. The remainder of the urine was acidified with phosphoric acid and extracted with ether containing 10 per cent of added alcohol. On evaporation of the ether the first crystals to be deposited melted at 188° and on recrystallization melted at 192–193°. The crystals showed all the properties of cinnamoylglycocoll, which will be described later. So far none of the operations had been conducted at a temperature of over 37°, so that it may be concluded that the cinnamoylglycocoll was actually present as such in the urine and was not the result of changes brought about outside the body. The bulk of the ethereal residue was worked up as follows: It was dissolved in hot water and distilled in steam to remove volatile acids; the solution was decolorized with a little charcoal, and filtered and concentrated. The crop of crystals deposited while the liquid was still somewhat warm was almost pure cinnamoylglycocoll,

[1] The phenylpropionic acid was tested and found to be free from cinnamic acid. When dissolved in sodium carbonate it did not decolorize potassium permanganate solution.

while much remained in the mother-liquor, together with hippuric acid. On recrystallization, 0.45 gram of pure cinnamoylglycocoll melting sharply at 192–193° and crystallizing in water in very long, thin, flat, glistening needles was obtained. The actual amount present in the urine must have been considerably larger.

Analysis:

0.1434 gm. substance gave 0.00952 gm. NH_3: N = 6.82 per cent.
$C_{11}H_{11}O_3N$ requires.....6.83 per cent.

The cinnamoylglycocoll on boiling with strong hydrochloric acid gave cinnamic acid, melting-point 132–133°. A solution of the substance in dilute sodium carbonate reduced potassium permanganate freely in the cold, with liberation of benzaldehyde. The substance was identical in every respect with the cinnamoylglycocoll prepared synthetically from cinnamoyl chloride and glycocoll and the melting-point of a mixture of the two specimens was unchanged.

The mother-liquor from the first crop of crystals on concentration furnished a deposit of semitranslucent short prisms melting indefinitely between 175–185°. The crystals were doubtless a mixture of hippuric acid with some cinnamoylglycocoll. After two additional crystallizations from water, pure hippuric acid (0.5 gram) melting sharply at 185–187° was obtained. It is easy to determine when all the cinnamoylglycocoll has been removed by dissolving a small portion of the crystals in dilute sodium carbonate and adding dilute potassium permanganate solution. Hippuric acid does not decolorize permanganate under these conditions, whereas cinnamoylglycocoll does so instantly with liberation of benzaldehyde. The test is best performed in a small open dish and serves for the detection of very small quantities of the cinnamoylglycocoll.

It must be mentioned that the isolation of cinnamoylglycocoll from the urine of cats that had received injections of sodium phenylpropionate was not always so successful as in the preceding case. In one case in which 2.5 grams of phenylpropionic acid were given to a cat weighing 3.1 kilos, the animal died after 40 hours, and although acetophenone (0.14 gram) and much hippuric acid were found in the urine, indications of only minute traces of cinnamoylglycocoll could be obtained.

In the urines from both the preceding experiments and also when 4.0 grams of phenylpropionic acid had been administered to a dog weighing 10 kilos, it was possible to obtain evidence of the probable presence of benzoylacetic acid. This was done by extracting a large quantity of the acidified urine with ether, filtering the emulsified ethereal layer and then allowing it to evaporate at the ordinary temperature. Part of the residue was dissolved in dilute alcohol and tested with ferric chloride. After the first formed precipitate of ferric hippurate had been filtered off, further addition of ferric chloride produced a deep violet purple color identical in every respect with that given by benzoylacetic acid under similar conditions. The criticism might justly be made that this reaction might equally well be due to acetoacetic acid. This is improbable, however, because no indications could be obtained of the formation of acetone on distilling the urines.

The urines from which it was possible to obtain qualitative reactions for benzoylacetic acid all contained considerable amounts of acetophenone. When but little or no acetophenone was present no reactions for benzoylacetic acid were ever obtained.

It is clear that the evidence for the occurrence of benzoylacetic acid in the urines investigated only warrants the deduction that the presence of this substance is probable. The amounts were too small for satisfactory purification and isolation.

Phenyl-β-oxypropionic acid was previously detected as an intermediate product in the catabolism of phenylpropionic acid[1] and was identified by conversion into cinnamic acid. It may also be readily detected as follows: The urine from cats or dogs which have received comparatively large doses of phenylpropionic acid (0.5 gram per kilo) is distilled to remove acetophenone and then extracted with ether in the manner previously described. The ethereal residue is taken up in water, distilled in steam, decolorized with charcoal and the aqueous solution concentrated and allowed to crystallize. The filtrate from the hippuric acid crystals is concentrated almost to dryness and the crystalline residue stirred up with about 3 cc. of ice-cold water. The aqueous solution is poured off and extracted by shaking with ether

[1] This *Journal*, iv, p. 426, 1908.

three times. The ether on evaporation usually leaves a syrup which occasionally will deposit crystals of phenyl-β-oxypropionic acid; as a rule, however, the product is still too impure. Phenyl-β-oxypropionic acid may be tested for as follows:

1. Part of the residue is boiled for a minute with a little strong hydrochloric acid. On cooling a crystalline deposit of cinnamic acid is obtained which on recrystallization melts at 132° and behaves in the characteristic fashion with alkaline permanganate solution.

2. Part of the syrup is diluted with water (30 cc.) and 1 cc. concentrated sulphuric acid added together with 5 cc. of 2 per cent potassium bichromate solution and the solution is then distilled. If all the chromic acid should appear to be reduced a further addition is made, but a very large excess should be avoided. The distillate contains acetophenone if phenyl-β-oxypropionic acid was present in the original solution. It is almost invariably accompanied by aldehyde, which must be removed before testing for the acetophenone. This is conveniently effected by adding a little ammoniacal silver nitrate to which caustic soda has been added, and then either warming to 35° for a short time or allowing the liquid to stand at the ordinary temperature for an hour or two. The liquid is next strongly acidified with phosphoric acid and redistilled. The first 2–3 cc. of the distillate is examined for acetophenone. Usually its characteristic smell is very evident and, unless very small quantities are present, the ketone is obtained in the form of oil drops. The comparatively small quantities of acetophenone are readily detected by the three following tests: (*a*) the formation of an insoluble paranitrophenylhydrazone, melting-point 184–5°; (*b*) the characteristic blue color obtained on adding dilute acetic acid drop by drop to the distillate after previously adding sodium nitroprusside and a little caustic soda; (*c*) the iodoform reaction. The first two of these tests are characteristic of acetophenone, the third is of course not characteristic but is the most sensitive and is useful for proving the absence of traces of acetophenone.

Another reaction for the detection of phenyl-β-oxypropionic acid which has sometimes been of use, although not so satisfactory as the preceding test, is based on the oxidation of the oxy-acid to the ketonic acid, benzoylacetic acid, the latter being identified by

its color-reaction with ferric chloride. The details of the test were identical with those employed by Black[1] in his reaction for the detection of β-oxybutyric acid. The ethereal residue previously referred to in which the phenyl-β-oxypropionic acid, if present, will be found, is neutralized by warming for several minutes with finely powdered barium carbonate. Complete neutralization is essential. The liquid is filtered, cooled and two drops of 2 per cent hydrogen peroxide added and then 5 per cent ferric chloride solution, containing a little ferrous salt, is added drop by drop. Usually a thick precipitate occurs at first and this must be removed by filtration. If phenyl-β-oxypropionic acid be present the filtrate on standing will turn a dark violet-red color which eventually turns almost black. The test cannot of course be applied in the presence of β-oxybutyric acid.

There has been no difficulty in obtaining all the foregoing reactions for phenyl-β-oxypropionic acid in urines from animals which have received doses of phenylpropionic acid corresponding to about 0.5 gram per kilo. The previous statements with regard to the occurrence of this substance have therefore received confirmation.

The fate of phenyl-β-oxypropionic acid. The additional data regarding the catabolism of this acid concern the determination of its greater resistance to oxidation in the body compared with phenylpropionic or cinnamic acid, and also the qualitative detection of benzoylacetic acid as an intermediary product of its oxidation in the body.

EXPERIMENT I. Three grams of the acid in the form of sodium salt were injected subcutaneously into a cat weighing 5.0 kilos. From the urine 2.35 grams of the unchanged acid were recovered by the methods previously described. The oxy-acid readily crystallized and after washing with chloroform and a little dry ether melted sharply at 93–94°. In addition about eight milligrams of acetophenone and 0.2 gram hippuric acid were separated. The reactions for benzoylacetic acid were doubtful. It will be seen therefore that oxidation under these conditions was very incomplete.

EXPERIMENT II. Two and one-half grams of acid, as sodium

[1]This *Journal*, v, p. 207.

salt, were given to a dog weighing 8.9 kilos. On examination the urine showed distinct traces of unchanged oxy-acid, which was lævorotatory (−0.72°) and was identified by all the reactions described on p. 210. The amount was quite small, however, —probably less than 0.2 gram. In addition acetophenone amounting to 0.0432 gram was found, together with much hippuric acid. The crude hippuric acid crystals weighed 0.9 gram and melted at 175–183°. On dissolving in sodium carbonate they reduced potassium permanganate with liberation of a little benzaldehyde, but the reduction was quantitatively slight. It is probable, therefore, that small quantities of cinnamoylglycocoll were present, but the amount was far too small to permit of satisfactory isolation. On recrystallization pure hippuric acid, melting-point 186.7°, was readily obtained. The reactions for benzoylacetic acid were obtained quite strongly.

Experiment III. Three grams of the acid were given to a dog weighing 10 kilos. The results were essentially similar to those described under Experiment II. (Acetophenone, 0.041 gram; hippuric acid, 1.0 gram; unchanged phenyl-β-oxypropionic acid, decided traces; cinnamoylglycocoll, possibly traces.)

Experiment IV. One and one-half grams were given to the same dog. The results were not materially different, although the quantity of acetophenone (0.02 gram) and of hippuric acid (0.07 gram) were naturally smaller. The quantity of unchanged oxy-acid was insignificant. The reactions for benzoylacetic acid were decidedly positive, but not so strong as in Experiment II.

The preceding results show that combustion of phenyl-β-oxypropionic acid is considerably more difficult than that of phenylpropionic acid or cinnamic acid. In doses of 0.6 gram per kilo, given to cats, the acid is scarcely attacked; given to dogs in doses of about 0.15–0.3 gram per kilo by far the greater part of the acid is burned.

The fate of cinnamic acid. Four grams of cinnamic acid were converted into the ammonium salt[1] and given subcutaneously to a dog weighing 17 kilos. The urine during the next 36 hours was collected, acidified with acetic acid and distilled. The dis-

[1] The sodium salt is inconvenient on account of its comparative insolubility.

tillate, which gave a strong iodoform reaction, was precipitated with paranitrophenylhydrazine acetate. 0.16 gram of hydrazone was obtained, equivalent to 0.08 gram of acetophenone. Additional amounts of hydrazone were obtained by repeating the injections of ammonium cinnamate and distilling the urine. On twice recrystallizing the crude nitrophenylhydrazone from alcohol it was eventually obtained in well-defined crystals melting at 183–184°. Part of the crystals were distilled with a little dilute sulphuric acid. The distillate contained oil drops possessing the characteristic smell of acetophenone and gave an intense positive reaction with sodium nitroprusside. There can be no question therefore of the excretion of acetophenone. Employing the usual methods, already described, a large quantity of hippuric acid crystals (1.5 grams) was obtained. The crude hippuric acid gave a decided reduction with permanganate in alkaline solution, with liberation of benzaldehyde, so that it is probable that it was contaminated with a little cinnamoylglycocoll. The quantity of the latter was too small to isolate, however. On recrystallization from water, pure hippuric acid, melting-point 185–187°, was readily obtained. The mother-liquor from the crude hippuric acid was examined for phenyl-β-oxypropionic acid in the usual way, with decided positive results with each test. The acid as usual appeared to be lævorotatory (−0.60°), but the quantity of substance was not large.

A similar experiment in which 2.0 grams were given to a cat of 4.6 kilos gave essentially similar results. The amount of acetophenone in the urine of the following 24 hours was 0.017 gram. Much hippuric acid (1.2 grams) and traces of phenyl-β-oxypropionic acid were also found. No unchanged cinnamic acid was found and only traces of cinnamoylglycocoll were present.

In another experiment ethyl cinnamate was given to a small cat (3.0 kilos) in amount corresponding to 1.0 gram of cinnamic acid per kilo. The animal survived—a result which indicates that cinnamic acid is less toxic than phenylpropionic acid. The results of the examination of the urine were similar to those of the preceding experiments. No benzoylacetic acid could be detected.

Fate of phenylpropionylglycocoll. The new data concerning the catabolism of this substance merely concern the demonstration

of the increased resistance to oxidation and diminished toxicity exhibited by the glycocoll derivative compared with phenylpropionic acid itself.

EXPERIMENT I. Three grams of phenylpropionylglycocoll prepared according to the method previously described[1] were converted into the sodium salt and given to a cat weighing 3.0 kilos. No toxic symptoms of any kind were noted. No acetophenone or hippuric acid could be detected in the urine passed during the next 24 hours, but 2.8 grams of unchanged phenylpropionylglycocoll were obtained from the ethyl acetate extract. The aqueous solution of the ethereal extract was optically inactive and no phenyl-β-oxypropionic acid could be detected. It may be safely concluded therefore that very little of the substance had been oxidized.

EXPERIMENT II. Four grams of phenylpropionylglycocoll were given to a dog weighing 9.0 kilos. A small quantity of acetophenone was detected (0.010 gram), but much less than would have followed a corresponding dose of phenylpropionic acid. All the available reactions for the detection of phenyl-β-oxypropionic acid (p. 210) were obtained. The rotation of the solution containing the phenyl-β-oxypropionic acid amounted to $-1.11°$. Strong indications of the presence of cinnamoylglycocoll were obtained with the permanganate test, but the substance could not be obtained in crystalline form. In addition the urine contained considerable amounts of unchanged phenylpropionylglycocoll, which by repeated crystallization was obtained in the form of good crystals melting at 113°. The amount of hippuric acid was not large enough to permit of its satisfactory separation by crystallization from the phenylpropionylglycocoll.

EXPERIMENT III. This experiment resembled Experiment II except that a smaller dose of phenylpropionylglycocoll (2.0 grams) was given to a larger dog (21 kilos). In this case the amount of unchanged phenylpropionylglycocoll was much smaller and the yield of hippuric acid correspondingly larger (0.9 gram). Acetophenone, phenyl-β-oxypropionic acid and cinnamoylglycocoll were readily identified.

Fate of phenyl-β-oxypropionylglycocoll. The results of experi-

[1] This *Journal*, iv, p. 431.

ments with this substance showed that it was scarcely attacked when given to either cats or dogs.

EXPERIMENT I. Three grams of the substance prepared according to the method already described[1] were converted into the sodium salt and injected into a cat weighing 3.1 kilos. On the following day a similar injection was made. The urine on distillation gave no trace of acetophenone, but a large quantity of unchanged phenyl-β-oxypropionylglycocoll was obtained from the ethylacetate extract. No hippuric acid could be detected. No toxic symptoms were shown at any time.

EXPERIMENT II. This experiment was similar to Experiment I except that smaller doses were employed. One and one-half grams were given to a cat weighing 4.1 kilos. Apart from a very doubtful trace of acetophenone, no substance other than unchanged phenyl-β-oxypropionylglycocoll was obtained from the urine.

EXPERIMENT III. Three grams of the substance converted into the sodium salt were given to a dog weighing 11 kilos. The distillate from the urine contained a trace of substance giving the iodoform reaction corresponding to 0.003 gram acetophenone. The quantity was too small to satisfactorily identify. No crystals of either hippuric acid or cinnamoylglycocoll could be obtained. The ethylacetate extract yielded almost 3.0 grams of syrupy substance which slowly crystallized and was identified as unchanged phenyl-β-oxypropionylglycocoll.

Fate of cinnamoylglycocoll. The experiments with this substance showed that, as in the case of the two other related derivatives, phenylpropionylglycocoll and phenyl-β-oxypropionylglycocoll, a greatly increased resistance to oxidation in the animal body is shown by the glycocoll derivative in comparison with the free acid.

Two grams of the substance prepared according to the method previously described[2] were converted into the sodium salt and administered to a cat weighing 4.7 kilos. The urine of the next 24 hours showed a minimal iodoform reaction, so that acetophenone, if present at all, could only have been present in insignificant

[1] This *Journal*, v, p. 201.
[2] This *Journal*, v, p. 303, 1908.

traces. The ethylacetate extract, after distillation in steam and treatment with charcoal, on filtering at once deposited a copious crop of glistening needles (1.1 grams), melting-point 192–193°, which proved to be unchanged cinnamoylglycocoll. On concentration of the mother-liquor two small additional crops of crystals were obtained, making in all 1.5 grams. No hippuric acid could be detected, so that the conclusion is reached that practically the whole cinnamoylglycocoll escaped oxidation and was excreted unchanged.

In another experiment in which the same dose was administered to a large dog (28 kilos), hippuric acid, melting-point 186–187°, was readily isolated.

SUMMARY.

Further experiments are described which throw some light upon the mode of conversion in the body of phenylpropionic acid into hippuric acid which extend and confirm the results previously obtained. Qualitative evidence has been obtained of the presence of benzoylacetic acid in the urine of cats which have received large doses of phenylpropionic acid (1.0 gram per kilo) and in addition the presence of phenyl-β-oxypropionic acid was confirmed. Two new tests for the detection of the latter substance are described, one depending upon its oxidation to acetophenone by means of chromic acid and the other upon its oxidation to benzoylacetic acid by hydrogen peroxide and ferric chloride. Acetophenone had already been satisfactorily isolated from the urines. These results harmonize well with the views previously advanced as to the mode of combustion of phenylpropionic acid in the body.

$$C_6H_5.CH_2.CH_2.COOH \rightarrow C_6H_5.CHOH.CH_2.COOH$$
$$\rightarrow C_6H_5.CO.CH_2.COOH \rightarrow C_6H_5.CO.CH_3$$
$$\searrow \qquad \swarrow$$
$$C_6H_5.COOH$$
$$\downarrow$$
$$C_6H_5.CO.NH.CH_2.COOH$$

In this scheme the probability is indicated of the conversion of part of the benzoylacetic acid into benzoic acid without going through the stage of acetophenone.

Under some conditions cinnamoylglycocoll, $C_6H_5.CH:CH.CO.NH.CH_2.COOH$, was isolated from the urine of animals that had received injections of sodium phenylpropionate and details of the detection and isolation of this substance are given. So far no definite conclusion has been reached as to the mode of formation of this substance in the body, but a number of possibilities are discussed. The evidence so far does not support the views that it originates either by the direct union of cinnamic acid and glycocoll or by the removal of the elements of water from phenyl-β-oxypropionylglycocoll, $C_6H_5.CHOH.CH_2.CO.NH.CH_2COOH$. It is doubtful if cinnamoylglycocoll is a normal intermediary product of the catabolism of phenylpropionic acid.

An investigation of the mode of combustion of cinnamic acid in the body showed that when the salts of this acid are administered subcutaneously to cats, phenyl-β-oxypropionic acid and acetophenone may be detected in the urine in addition to hippuric acid—the latter substance being the normal end-product of the reaction. These results indicate that the unsaturated cinnamic acid, at least in part, takes up the elements of water to give phenyl-β-oxypropionic acid and that the latter yields benzoic (hippuric) acid through the stage of benzoylacetic acid and acetophenone in accordance with the results of the experiments upon the fate in the body of phenyl-β-oxypropionic acid itself. It is not improbable that the conversion of cinnamic acid into phenyl-β-oxypropionic acid may be a reversible reaction and may be of importance as illustrating a type of change which would account for the production of unsaturated acids from saturated acids through the medium of the corresponding β-oxy-acid and also the reverse change.

The probable mode of combustion in the body of cinnamic acid is represented on p. 207.

Additional experiments upon the fate of phenyl-β-oxypropionic acid are recorded. Qualitative evidence was obtained of the presence of benzoylacetic acid in the urine, when a sufficient quantity of the β-oxy-acid was administered to dogs.

Details are recorded of experiments upon the fate of the glycocoll derivatives of phenylpropionic acid, phenyl-β-oxypropionic acid and cinnamic acid, in which it is shown that these substances are far less readily attacked in the organism, at least

in the case of cats and dogs, than are the salts of the free acids uncombined with glycocoll.

Note added during Proof Correction. My attention has been drawn to the fact that the cinnamoylglycocoll described in this and other papers has already been obtained by E. Fischer and P. Blank from the products of the action of ammonia upon β-phenyl-α-brompropionylglycocoll. *Annalen der Chemie*, cccIix, p. 1.

THE MODE OF OXIDATION IN THE ANIMAL ORGANISM OF PHENYL DERIVATIVES OF FATTY ACIDS. PART V.

STUDIES ON THE FATE OF PHENYLVALERIC ACID AND ITS DERIVATIVES.

By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, April 24, 1909.)

The main point of interest in studies upon the fate in the animal organism of phenyl derivatives of fatty acids concerns the relation which the results bear to fatty acid metabolism in general. Investigations of the mode of oxidation of phenyl derivatives with side chains containing three and four carbon atoms have already been made and it therefore remained to try to trace some of the steps in the catabolism of acids with five carbon atoms in their side chain. The results of such an investigation are recorded in the following paper. Since the theoretical possibilities for the occurrence of different types of oxidation are greater than in the case of the acids previously studied, several new factors require consideration.

The only acid of the series in question whose fate in the animal body has been determined is phenylvaleric acid. This substance was fed to dogs by Knoop[1] and he observed a subsequent excretion of hippuric acid. This result was of great significance when contrasted with his observations on the excretion of phenaceturic acid following the administration of phenylbutyric acid. In Knoop's paper no attempt is made to picture the mechanism of the reaction. The acid is stated to be "oxidiert am δ C-atom,"

$$C_6H_5.CH_2.CH_2.CH_2.CH_2.COOH \rightarrow C_6H_5.COOH \rightarrow C_6H_5.CONH.CH_2.COOH$$

[1] *Der Abbau aromatischen Fettsäuren im Tierkörper*, Freiburg, Ernst Kuttraff, 1904.

but it is clearly to be inferred that Knoop recognized the possibility of the oxidation being indirect, i.e., that the oxidation at the δ carbon atom was not the primary process.

A further study of the fate of phenylvaleric acid, when administered by subcutaneous injection in relatively large doses to cats, has clearly shown that the conversion of phenylvaleric acid into hippuric acid is an indirect process, i.e., the aliphatic side chain is not primarily oxidized in the δ position. The evidence for this belief is based on the detection of phenyl-β-oxypropionic acid, cinnamoylglycocoll and acetophenone in the urine of animals that had received injections of sodium phenylvalerate in doses of about 0.8 gram per kilo. These substances are all intermediary products in the catabolism of phenylpropionic acid and by their further oxidation in the body yield hippuric acid. It is therefore probable that the phenylvaleric acid primarily undergoes oxidation so as to yield phenylpropionic acid, so that the original side chain of the phenylvaleric acid with five carbon atoms is converted into phenylpropionic acid with a three-carbon side chain, and this by further oxidation into benzoic acid with a one-carbon side chain.

$$C_6H_5.CH_2.CH_2.CH_2\left|CH_2.COOH\rightarrow C_6H_5\right|CH_2.CH_2.COOH\rightarrow C_6H_5.COOH$$

Judging by analogy with other similar reactions[1] it was probable that the conversion of phenylvaleric acid into phenylpropionic acid would take place through the intermediate formation of phenyl-β-oxyvaleric acid, and this was made still more probable through the observation that phenyl-β-oxyvaleric acid, on administration to cats under the same conditions as those employed in the case of phenylvaleric acid, resulted in the excretion of hippuric acid with the same intermediary products as were detected in the latter case, namely, phenyl-β-oxypropionic acid, acetophenone and cinnamoylglycocoll. *It is probable, therefore, that phenyl-β-oxyvaleric acid represents the first step in the catabolism of phenylvaleric acid.* It is theoretically probable that the corresponding β-ketonic acid would represent the next stage in the

[1] Such as the conversion of phenylbutyric acid into phenaceturic acid through phenyl-β-oxybutyric (this *Journal*, v, p. 173) and the oxidation of phenylpropionic acid to hippuric acid through phenyl-β-oxypropionic acid.

oxidation, but of this no proof is forthcoming. That the β-ketonic acid, if formed, does *not* part with carbon dioxide, so as to yield the corresponding ketone,[1]

$$C_6H_5.CH_2.CH_2.CO.CH_2.COOH \rightarrow C_6H_5.CH_2.CO.CH_3$$

was proved by showing that the ketone benzylacetone is oxidized in the animal body to phenylacetic acid, which is excreted in the usual way as phenaceturic acid:

$$C_6H_5.CH_2.CH_2.CO.CH_3 \rightarrow C_6H_5.CH_2.COOH \rightarrow C_6H_5.CH_2.CONH.CH_2.COOH$$

This reaction is analogous to the oxidation of phenylacetone, which was shown to yield hippuric acid when administered to a dog.[2]

If the β-ketonic acid, $C_6H_5.CH_2.CH_2.CO.CH_2.COOH$, actually represents the first stage in the oxidation of phenyl-β-oxyvaleric acid, it is much more probable that this substance undergoes oxidation or hydrolysis so as to yield phenylpropionic acid with loss of two carbon atoms. Such a reaction is thoroughly in accord with the behavior of β-ketonic acids on oxidation or hydrolysis *in vitro*, and similar reactions are known to occur in the body. Thus it was found that benzylaceto-acetic ester, when administered to cats or dogs, gave *hippuric acid*, a result which is most readily explained on the assumption that phenylpropionic acid or a derivative of this acid is first formed, which is then oxidized to benzoic acid and excreted as hippuric acid in the usual way:

$$CH_3.CO.CH(CH_2.C_6H_5).COOH \rightarrow C_6H_5.CH_2.CH_2.COOH \rightarrow C_6H_5.COOH$$
$$\rightarrow C_6H_5.CO.NH.CH_2.COOH$$

With the assumption that phenylpropionic acid is formed from phenyl-β-oxyvaleric acid, with the possible intermediary formation of phenylpropionylacetic acid, the remaining steps in the catabolism of phenylvaleric acid will be identical with those

[1] A reaction analogous to the conversion of aceto-acetic acid into acetone.
[2] This *Journal*, v, p. 183.

of phenylpropionic acid. The whole series of changes may be represented as follows:

$C_6H_5.CH_2.CH_2.CH_2.CH_2.COOH$
(Phenylvaleric acid)
↓
$C_6H_5.CH_2.CH_2.CHOH.CH_2.COOH$
(Phenyl-β-oxyvaleric acid)
↓
$[C_6H_5.CH_2.CH_2.CO.CH_2.COOH]$?
(Phenylpropionylacetic acid)
↓
$C_6H_5.CH_2.CH_2.COOH$
(Phenylpropionic acid)
↓
$C_6H_5.CHOH.CH_2.COOH$ (phenyl-oxypropionic acid) ⇄ $C_6H_5.CH:CH.COOH$ (Cinnamic Acid)
↓ ↓
$C_6H_5.CO.CH_2.COOH$ (Benzoylacetic acid) $C_6H_5.CH.CH.CO.NHCH_2.COOH$[1] (Cinnamoylglycocoll)
↓
$C_6H_5.CO.CH_3$
(Acetophenone)
↓
C_6H_5COOH
(Benzoic acid)
↓
$C_6H_5.CO.NH.CH_2.COOH$
(Hippuric acid)

In order to determine whether this mode of oxidation of the side chain of phenylvaleric acid by which the four carbon groups are removed in two pairs, constituted a general type of reaction it was decided to investigate the fate of a number of derivatives of phenylvaleric acid. The substances examined were as follows:

Phenyl-α-β-pentenic acid	$C_6H_5.CH_2.CH_2.CH:CH.COOH$
Phenyl-β-γ-pentenic acid	$C_6H_5.CH_2.CH:CH.CH_2.COOH$
Cinnamylidene acetic acid	$C_6H_5.CH:CH.CH:CH.COOH$
Phenyl-γ-oxyvaleric acid	$C_6H_5.CH_2.CHOH.CH_2.CH_2.COOH$
Cinnamylidenemalonic acid	$C_6H_5.CH:CH.CH:C(COOH)_2$

[1] The mode of formation of cinnamoylglycocoll is not clear. A discussion of this question is contained in the preceding paper (p. 204); it probably is not derived from the direct coupling of glycocoll and cinnamic acid as represented in the diagram which is intended merely to show the structural relations of the substance.

Of these substances the first three were oxidized to benzoic acid and excreted as hippuric acid in the urine. In the case of each of these substances evidence was obtained that the oxidation took place in such a fashion that the five-carbon atom side-chain was converted primarily into a three-carbon atom side-chain and the latter again oxidized with a further loss of two carbon groups. Acetophenone and phenyl-β-oxypropionic acid, and probably cinnamoylglycocoll, were detected in the urine in each case. *The mode of oxidation of these three substances is completely analogous to that of phenylvaleric acid and phenyl-β-oxyvaleric acid.*

The two remaining substances, phenyl-γ-valeric acid and cinnamylidenemalonic acid, were scarcely attacked when administered to cats, for the greater part was excreted unchanged. The resistance of phenyl-γ-valeric acid to oxidation in the body is analogous to the similar behavior of phenyl-γ-oxybutyric acid. It appears that γ-oxy-acids are commonly oxidized in the body with difficulty and appear to be converted into lactones and excreted in the urine. The lactones as a class are much more resistant to oxidation *in vitro* than are the oxy-acids from which they are derived through loss of a molecule of water.

To sum up: evidence has been obtained that five acids of the type Ph. C.C.C.C.C.COOH undergo oxidation in the body in such a way that four carbon atoms are removed from the side chain in *two pairs*. In every case benzoic acid was the end product.

$$\text{Ph. C.} \mid \text{C.C.} \mid \text{C.COOH} \rightarrow \text{Ph. C.} \mid \text{C.COOH} \rightarrow \text{Ph.COOH}$$

This type of oxidation may be termed successive β-oxidation, and I see no reason to suppose that it is not a general biochemical reaction. Assuming that this is so, a ready explanation may be given of the extraordinarily interesting results obtained by Embden[1] and his co-workers through the perfusion of surviving livers with salts of fatty acids. He finds that of the normal fatty acids, butyric, valeric, caproic, heptylic, octylic, nonylic and decoic, those and only those with an even number of carbon atoms yield aceto-acetic acid and acetone. These substances

[1] Hofmeister's *Beiträge*, viii, pp. 121, 129; xi, p. 318.

are undoubtedly derived from β-oxybutyric acid and the latter from butyric acid. Hitherto there has been no evidence to decide whether the conversion of an acid such as decoic acid into butyric acid which necessitates the removal of six carbon atoms is effected in one step or is due to successive removal of two or some multiple of two carbon atoms at a time. *The results obtained with the derivatives of phenylvaleric acid make it very probable that the catabolism of a fatty acid group,* $-CH_2.(CH_2)_n.COOH$, *is effected by the successive removal of two carbon groups at a time*, and Embden's results are in complete harmony with this view.

If this hypothesis be essentially correct it follows that ordinarily only one molecule of either β-oxybutyric acid, aceto-acetic acid, or acetone, can result from the catabolism of one molecule of a fatty acid such as stearic acid. On the other hand it must be recalled that the synthetic formation of aceto-acetic acid from simpler substances containing two carbon atoms appears probable.[1] The observed excretion of acetophenone and phenyl-β-oxypropionic acid following the administration of cinnamylideneacetic acid is of interest when compared with the similar excretion of these substances when cinnamic acid is injected. It is probable that the cinnamylideneacetic acid is oxidized through the stage of cinnamic acid through β-oxidation and that a second β-oxidation converts the latter into benzoic acid which is excreted in the form of hippuric acid.

$$C_6H_5.CH:CH:\vdots CH.COOH \rightarrow C_6H_5.CH:\vdots CH.COOH \rightarrow C_6H_5:COOH$$

EXPERIMENTAL.

Preparation of phenylvaleric acid. The phenylvaleric acid used in the following experiments was obtained by the following series of reactions. Cinnamic aldehyde was condensed with malonic acid in the presence of a trace of aniline. The resulting cinnamylidenemalonic acid was reduced to phenylpropenyl-malonic acid with sodium amalgam according to Thiele and Meisenheimer's method,[2] and on boiling with water gave phenyl-

[1] Friedmann: Hofmeister's *Beiträge*, xi, p. 202.

[2] *Annalen der Chemie*, cccvi, p. 247.

β-γ-pentenic acid. The latter substance was partially converted into phenyl-α-β-pentenic acid[1] by boiling with caustic soda as described by Fittig and Hoffmann[2] and the latter reduced with sodium amalgam to the desired phenylvaleric acid. The changes may be represented as follows:

$$C_6H_5.CH{:}CH.CHO + CH_2.(COOH)_2 \rightarrow C_6H_5.CH{:}CH.CH{:}C(COOH)_2 \rightarrow$$
$$C_6H_5.CH_2.CH{:}CH.CH.(COOH)_2 \rightarrow C_6H_5.CH_2.CH{:}CH.CH_2.COOH \rightarrow$$
$$C_6H_5.CH_2.CH_2.CH{:}CH.COOH \rightarrow C_6H_5.CH_2.CH_2.CH_2.CH_2.COOH$$

The phenylvaleric acid crystallized in glistening platelets, melting point 58–59°, and its properties in every way agreed with those described by Fittig and Hoffmann. The practical details of the preparations with the exception of that of cinnamylidenemalonic acid are omitted, since they may be found in the various references given and only immaterial modifications of the original methods were made.

The employment of cinnamylidenemalonic acid instead of cinnamylideneacetic acid as starting material for the synthesis of phenylvaleric acid has very decided advantages, for it is obtained in better yield from the cinnamic aldehyde from which they are both prepared and in addition it is more readily reduced with sodium amalgam. Cinnamylidenemalonic acid has hitherto been prepared by either heating cinnamic aldehyde with malonic acid in the presence of acetic acid or by Knoevenagel's method, in which 1–2 molecules of alcoholic ammonia is used to effect the condensation between the aldehyde and malonic acid. It was found that a preferable method was to dissolve malonic acid (1 molecule) in a minimum amount of hot 90 per cent alcohol, then add cinnamic aldehyde (1 molecule) and a drop or two of aniline, and let the whole stand for several hours at the ordinary temperature. On addition of the aniline, the solution becomes dark orange colored and after a few minutes crystals of cinnamylidenemalonic acid begin to separate and finally the whole mixture becomes almost solid. The mass is then ground up with 90 per cent alcohol and well drained, using suction. The cinnamylidenemalonic acid is almost pure and after a single

[1] Phenyl-β-oxyvaleric acid is formed simultaneously.

[2] *Annalen der Chemie*, cclxxxiii, p. 314.

crystallization from alcohol melts at 208–210°. The yield is about 70 per cent of theory when using quantities of about 50 grams of material, at each operation.

Fate of phenylvaleric acid. Phenylvaleric acid (3.2 grams) was exactly neutralized with aqueous caustic soda, using a trace of phenolphthalein as indicator and the sterile solution given subcutaneously to a cat (4.1 kilos), the urine being collected during the following forty-eight hours. The first portion of urine collected gave on distillation a strong iodoform reaction. The urines before the injection and on the second day afterwards gave practically negative results. The iodoform reaction was undoubtedly mainly, if not exclusively due to acetophenone. The distillate was treated with a cold filtered solution of para-nitrophenylhydrazine acetate. A turbidity at once appeared and on cooling the solution for a short time in ice a small but definite precipitate was obtained. The precipitate was filtered off and washed with cold water. As the amount was too small to purify by recrystallization the precipitate was distilled from a small flask with 10 per cent sulphuric acid. The first few drops of the distillate smelt of acetophenone, and, in addition to the iodoform reaction, gave the characteristic reaction with sodium nitroprusside.

The urine after distillation was concentrated, acidified with phosphoric acid and extracted in the usual way[1] with ether containing a little alcohol. The extract was steam-distilled and the solution after treatment with charcoal was concentrated and allowed to crystallize. The crystals which separated melted indefinitely between 165° and 180° and proved to be a mixture of cinnamoylglycocoll and hippuric acid. The two substances were separated by fractional crystallization from boiling water in the manner described in the preceding paper. The cinnamoylglycocoll separated first from the warm solution in long needles which were purified by recrystallization. The amount of pure crystalline cinnamoylglycocoll recovered was 0.17 gram. The loss during precipitation must have been very considerable. The cinnamoylglycocoll was identified by its crystalline form, (melting point 192–193°), by the formation of cinnamic acid

[1] The details of the methods of urine analysis were identical with those previously employed and described.

(melting point 132.3°)on hydrolysis with concentrated hydrochloric acid, by its oxidation to benzaldehyde on treating the alkaline solution in the cold with dilute potassium permanganate and by the following analysis.

0.1497 gram gave NH_3 = 0.01036 gm. (Kjeldahl): N = 6.92 per cent
$C_{11}H_{11}O_3N$ requires 6.83 per cent.

The melting point of the substance when mixed with cinnamoylglycocoll prepared synthetically was unchanged.

On evaporating the mother liquor from the first crystallization through which most of the cinnamoylglycocoll has been removed, crystals were obtained which gave pure hippuric acid, melting point 186.7°, after two additional crystallizations.

Analysis:
0.1510 gm. gave NH_3 = 0.0119 gm. (Kjeldahl): N = 7.88 per cent
$C_9H_9O_3N$ requires 7.82 per cent N.

The mother liquor from the hippuric acid crystals was examined in the way described in the previous paper (p. 210) for phenyl-β-oxypropionic acid. Decided qualitative evidence of its presence was obtained as follows: The aqueous solution of the ethereal extract was laevorotatory (0.32°), it gave acetophenone when oxidized with chromic acid and cinnamic acid, melting point 131°–132°, when hydrolyzed with hydrochloric acid. The amount of oxy-acid was not large.

Two similar experiments were made in which sodium phenylvalerate was given to cats. In one case approximately the same dose was employed (0.7 gram per kilo) and the results were identical with the foregoing except that the cinnamoylglycocoll could not be isolated in crystalline form. Decided qualitative evidence of its presence was obtained, however. In the other case 1.0 gram of phenylvaleric acid was given in the form of the sodium salt to a cat weighing 4.7 kilos. Almost the whole of the substance was converted into hippuric acid.

Phenyl-β-oxyvaleric acid. This acid was prepared by boiling phenyl-β-γ-pentenic acid for five days with 10 per cent caustic soda (10 molecules) according to Fittig and Hoffmann's method.[1] It was separated from the other acids by crystallization from

[1] *Annalen der Chemie*, cclxxxiii, p. 313.

carbon bisulphide. The acid was obtained in very well formed crystals, melting point 131°.

EXPERIMENT I. Three and one-half grams of the acid were neutralized with caustic soda solution and injected subcutaneously into a female cat weighing 4.5 kilos. The urine (48 hours) was analyzed exactly as in the preceding case. On distillation decided evidence of the presence of acetophenone was obtained. The ether extract after crystallization gave a considerable amount of unchanged phenyl-β-oxyvaleric acid, so the whole extract was neutralized with soda and injected into another cat (5 kilos). The urine formed during the next 48 hours was again analyzed. The results were entirely similar to those obtained in the case of phenylvaleric acid, although the amount of cinnamoylglycocoll was unfortunately too small for analysis. It was readily identified, however, by its melting point, 192–193°, by the reaction with potassium permanganate and by the formation of cinnamic acid (melting point 132°) on hydrolysis with hydrochloric acid. The hippuric acid was recrystallized repeatedly until it no longer reduced permanganate in alkaline solution. 0.6 gram of the pure substance, melting point 186–187°, was obtained.

Analysis.

0.2000 gram gave NH_3 = 0.0156 gm. N = 7.80 per cent
$C_9H_9O_3N$ requires 7.82 per cent.

Some of the qualitative reactions for phenyl-β-oxypropionic acid which were obtained were invalidated owing to the probable presence of unchanged phenyl-β-oxyvaleric acid, but the formation of acetophenone on oxidation with chromic acid must be regarded as fair presumptive evidence of its presence. In addition to the foregoing another experiment was made in which 1.7 grams were given to a cat weighing 4 kilos. The results were similar except that no cinnamoylglycocoll could be detected. Much hippuric acid (0.5 gram) was obtained.

Phenyl-α-β-pentenic acid. This acid was obtained as a by-product in the preparation of phenyl-β-oxyvaleric acid. The acid was crystallized from ether and melted at 104° and exactly corresponded with Fittig and Hoffmann's description of the acid. Three grams of the acid were converted into the sodium salt and

given subcutaneously to a cat weighing 3.1 kilos. The urine during the next twenty-four hours contained acetophenone (=0.020 gram) and gave a strong positive nitroprusside reaction for that substance when the distillate was treated as described in the case of phenylvaleric acid. The ethyl acetate extract of the acidified urine gave much hippuric acid which after recrystallization melted sharply at 185–187°. No unchanged phenylpentenic acid was detected. Qualitative indications of the probable presence of cinnamoylglycocoll were obtained with the permanganate reaction, but none could be obtained in crystalline form free from hippuric acid, and the amount of the substance present was therefore small. All the reactions for phenyl-β-oxypropionic acid were obtained. The ethereal extract had a laevorotation of 0.22° and gave acetophenone in easily recognizable quantities when oxidized with chromic acid.

Phenyl-β-γ-pentenic acid. This acid was prepared by the reduction of cinnamylidenemalonic acid with sodium amalgam and boiling the reduction product with water which removes carbon dioxide. Three grams of the acid were neutralized with soda and given to a cat weighing 4.6 kilos. The animal experienced slight toxic symptoms but rapidly recovered. The urine passed during the next 48 hours contained 0.045 gram acetophenone which was readily identified on redistillation. The ethyl acetate extract on crystallization gave crystals melting indefinitely about 175–182° which were doubtless a mixture of hippuric acid and cinnamoylglycocoll. The crystals gave an abundant yield of benzaldehyde on treatment with permanganate, but on repeated recrystallization from water only hippuric acid could be obtained in the pure crystalline condition, melting point 186.7°. The "ethereal extract" from the hippuric acid mother liquor was laevorotatory (0.32°) and gave all the qualitative reactions for phenyloxypropionic acid, including the formation of acetophenone on oxidation with chromic acid, the production of cinnamic acid on treatment with hydrochloric acid, and the reaction with hydrogen peroxide and ferric chloride. In a second experiment 4.5 grams were given to a cat weighing 4.0 kilos. This dose proved fatal, the animal dying in about twenty hours. The urine contained 0.015 gram of acetophenone, but no benzoylacetic acid could be detected.

Phenyl-γ-oxyvaleric acid. The lactone of this acid was prepared by the action of sodium carbonate on phenyl-γ-bromovaleric acid according to Fittig and Stern's method.[1] Two grams of the lactone were converted into the sodium salt by warming with an excess of caustic soda. The excess of the latter was neutralized exactly with hydrochloric acid and the sterile solution injected subcutaneously into a cat weighing 4.0 kilos. No toxic symptoms followed the injection. The ether extract, after steam distillation, yielded 0.9 gram of an unchanged lactone which was extracted with chloroform. The lactone was identified by conversion into the silver salt which was analyzed. No hippuric acid or other products were detected.

Cinnamylideneacetic acid. The substance was prepared by Riedel's method by the condensation of malonic acid with cinnamic aldehyde by means of pyridine at the boiling temperature.[2] Three grams of the acid were neutralized with soda and injected into a cat weighing 4.1 kilos. The animal showed signs of poisoning after an hour and died some fourteen hours afterwards. In another experiment two grams were given to a small dog weighing 4.9 kilos. The urine passed during the next forty-eight hours contained 0.028 gram of acetophenone. The results of the analysis of the urine were identical with those in the case of phenyl-β-γ-pentenic acid.

Cinnamylidenemalonic acid. Two grams of this acid prepared as previously described were converted into the sodium salt and given to a cat (4.5 kilos). No acetophenone could be detected in the urine and on acidifying with sulphuric acid practically the whole of the acid was precipitated unchanged.

Benzylacetone. The substance was prepared by boiling benzylaceto-acetic ester (25 grams) with caustic soda (25 grams) and water (350 cc.) for several hours. The oily product was then distilled and the fraction boiling between 230–240° was separately collected. The yield was 12.0 grams.

For purposes of identification the paranitrophenylhydrazone of the ketone was prepared according to the usual method. The substance crystallizes from hot 95 per cent alcohol in long, well-formed needles, often grouped in rosettes, melting sharply at

[1] *Annalen der Chemie*, cclxviii, p. 94.
[2] *Annalen der Chemie*, ccclxi, p. 96, 1908.

110–111°. Three grams of the ketone were diluted with an equal volume of alcohol and injected into a cat weighing 2.55 kilos. Toxic symptoms set in during the next two hours and the animal died after being in a comatose state for almost thirty-six hours. The urine contained a little unchanged ketone. In another experiment 2.5 grams were given to a dog weighing 25 kilos. The urine gave very little unchanged ketone on distillation and on extraction with ethyl acetate a good yield (0.9 gram) of phenaceturic acid was obtained by the usual methods.

Benzylacetoacetic ester. This substance was prepared according to Conrad and Bischoff's method.[1] Three grams of the ester dissolved in 3.0 grams of diluted alcohol were injected subcutaneously into a cat weighing 4.9 kilos. On distilling the urine passed during the next sixty hours there was obtained less than 0.1 gram of ketone derived from unchanged ester. The urine was extracted with ethyl acetate and after analyzing in the usual way 0.6 gram of pure hippuric acid, melting point 186.7° was obtained. No indication was obtained of the presence of phenaceturic acid.

[1] *Annalen der Chemie*, cciv, p. 179.

THE MODE OF OXIDATION IN THE ANIMAL ORGANISM OF PHENYL DERIVATIVES OF FATTY ACIDS. PART VI.

THE FATE OF PHENYLALANINE, PHENYL-β-ALANINE, PHENYLSERINE, PHENYLGLYCERIC ACIDS AND PHENYLACETALDEHYDE.

By H. D. DAKIN.

(*From the Laboratory of Dr. C. A. Herter, New York.*)

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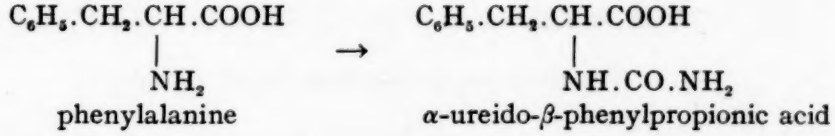
As is well known phenylalanine, tyrosine and phenyl-α-oxypropionic acid are apparently completely oxidized when introduced into the animal body. The behavior of these α-substitution derivatives of phenylpropionic acid is in marked contrast to that of phenylpropionic acid itself and of its β-substitution derivatives, and so far the cause of this difference has received no adequate explanation.

In the effort to collect data which might throw light upon this subject it was deemed of interest to investigate the fate of some substances related to both classes of compounds in order to try to determine some of the relations between structure and the mode of catabolism in the animal organism. In addition new experiments were made upon the fate of phenylalanine itself which resulted in the discovery of a new substance the constitution of which may furnish a clue to some of the biochemical reactions which this substance and other related α-amido-acids undergo.

Numerous experiments have been made in which small or moderate doses of phenylalanine have been administered to animals, and it is generally agreed that under these conditions the substance undergoes complete decomposition with elimination of the nitrogen in the form of urea. My own experiments were made by injecting *r*-phenylalanine dissolved in warm salt solution into the femoral vein of cats. Six to eight grams of phenyl-

alanine were used and the volume of solution was about 300 cc. Urine is of course rapidly secreted during the injection and during the following hours. On examination the urine was found to contain a considerable amount of unchanged *r*-phenylalanine, together with a crystalline substance which was separated and identified as *α-ureido-β-phenylpropionic acid.*

For comparison this substance was obtained synthetically by the action of potassium cyanate upon phenylalanine and proved to be identical in every respect with the substance isolated from the urine.



The question at once arises as to whether the formation of this urea derivative represents the primary stage in the normal catabolism of phenylalanine. It is commonly assumed that ammonia is liberated from the α-amino-acids and that it is converted into urea in the liver, but from the preceding result it would certainly appear as if urea formation might occur without prior separation of the nitrogen in the form of ammonia.[1] The production of α-uramido-β-phenylpropionic acid from phenylalanine recalls the similar production of urea derivatives from meta-amidobenzoic acid,[2] taurine[3], tyrosine[4] and a number of other aromatic substances. Further experiments will be made to try to discover whether the formation of α-uramido-β-phenylpropionic acid from phenylalanine represents a general type of biochemical reaction. The reaction has an interesting bearing upon Hofmeister's and other allied theories of urea formation.

Although phenylalanine was injected in such large amounts that much was excreted unchanged, no evidence could be obtained of the presence in the urine of phenyl-α-oxypropionic acid,

[1] The possibility of the uramido-acids playing a part in the mechanism of urea formation has been referred to by F. Lippich (*Ber. d. deutsch. chem. Ges.*, xli, pp. 2953, 2974). No experiments were cited however.

[2] E. Salkowski: *Zeitschr. f. physiol. Chem.*, vii, p. 113.

[3] E. Salkowski: *Ber. deutsch. chem. Ges.*, vi, p. 744.

[4] Blendermann: *Zeitschr. f. physiol. Chem.*, vi, p. 253.

phenaceturic acid, homogentisic acid or other phenolic derivative.

The fate of phenyl-β-alanine, $C_6H_5.CH.NH_2.CH_2.COOH$, was next investigated. This substance was administered to cats and dogs by subcutaneous injection of aqueous solutions and underwent a series of reactions entirely different to those of the isomeric phenyl-α-alanine. The following substances were found in the urines of the injected animals: phenyl-β-oxypropionic acid, acetophenone and hippuric acid. There can be little doubt but that the following scheme represents the mode of breakdown of phenyl-β-alanine.

$C_6H_5.CHNH_2.CH_2.COOH$
(phenyl-β-alanine)
↓
$C_6H_5.CHOH.CH_2.COOH$
(phenyl-β-oxypropionic acid)
↓
$C_6H_5.CO.CH_2.COOH$
(benzoylacetic acid)
↓
$C_6H_5.CO.CH_3$
(acetophenone)
↓
$C_6H_5.COOH$
(benzoic acid)
↓
$C_6H_5.CO.NH.CH_2.COOH$
(hippuric acid)

The first step in this series of changes, involving the replacement of an (NH_2) group by an (OH) group is analogous to the formation of mandelic acid from phenyl-α-amidoacetic acid and of lactic acid from alanine. The steps in the further oxidation of the resulting phenyl-β-oxypropionic acid, resulting in the formation of hippuric acid as the end product, follow from the previous investigations[1] upon the fate in the body of the former substance.

[1] This *Journal*, iv, p. 428, vol. vi, p. 203.

There is therefore the greatest contrast between the biochemical reactions of the two isomeric phenylalanines, and the results furnish an additional example of the great difference in the changes which the α and β-substitution derivatives of phenylpropionic acid are prone to undergo in the animal body.

Judging from the fact that phenylalanine, phenyl-α-oxypropionic acid and phenylpyruvic acid are all apparently capable of complete oxidation in the animal organism, it might be inferred that the presence of the α-substituted group, C:CHR.COOH, constituted the condition necessary for the aromatic nucleus of the derivatives of phenylpropionic acid to be capable of oxidation in the body. That this condition does not necessarily have this effect is shown by the results of the investigation of the fate of phenylserine, $C_6H_5.CHOH.CHNH_2.COOH$ and of the two isomeric phenylglyceric acids, $C_6H_5.CHOH.CHOH.COOH$.

Phenylserine was found to undergo β-oxidation when administered to cats; the benzoic acid thus formed being excreted as hippuric acid in the usual way:

$$C_6H_5.CHOH.CHNH_2.COOH \rightarrow C_6H_5.COOH \rightarrow C_6H_5.CO.NHCH_2.COOH$$

It is remarkable that two such closely allied substances as phenylserine and phenylalanine should undergo such widely different reactions in the body.

The phenylglyceric acids when administered to cats and dogs proved to be somewhat difficult of attack, but both of them underwent β-oxidation with formation of benzoic acid, which was excreted as hippuric acid:

$$C_6H_5.CHOH.CHOH.COOH \rightarrow C_6H_5.COOH \rightarrow C_6H_5.CONH.CH_2.COOH$$

It is therefore clear that neither a $CHNH_2.COOH$ group such as is present in phenylalanine nor CHOH.COOH group such as phenyl-α-oxypropionic acid is able to confer on a substance the property of undergoing complete oxidation in the animal body if a second hydroxyl or amino group be present in the β-position.

The results also show clearly that neither phenylalanine nor phenyl-α-oxypropionic acid undergo β-oxidation in the body, since, if this were the case, hippuric acid would be found among the products of their catabolism. The same conclusion is doubtless true in the case of tyrosine.

In addition, it may be fairly inferred that when an α-β-di-substituted phenylpropionic acid derivative undergoes catabolism the β-position is the preferred point of attack.[1] In relation to this conclusion, it is interesting to recall the fact that phenyl-β-γ-dioxybutyric acid, is converted into hippuric acid in the body, oxidation taking place at the γ-position rather than the β-position.[2]

In trying to picture the steps in the oxidative breakdown of phenylalanine, two alternatives demand special consideration, since β-oxidation is excluded. These are oxidation in the nucleus or α-oxidation in the side chain. There is some evidence based upon L. Blum's[3] striking experiments upon the formation of homogentisic acid, leading to the belief that the two processes may occur simultaneously. If this were not the case one might anticipate that the first step in the α-oxidation of phenylalanine would be phenylacetaldehyde.[4]

$$C_6H_5.CH_2.CHNH_2.COOH \rightarrow C_6H_5.CH_2.CHO + NH_3 + CO_2$$

Direct experiment showed that phenylacetaldehyde when administered to dogs, was at least in part, converted into phenaceturic acid. It is therefore improbable that phenylacetaldehyde is a product of the catabolism of phenylalanine, although it is of course apparent that the type of experiment just cited cannot be held to furnish absolute proof of such a statement.

In connection with the question of the mode of breakdown of the aromatic nucleus of substances such as phenylalanine when introduced into the body, I wish to refer to some results which I hope to amplify in the future. It has been found that phenylalanine, and a number of other aromatic acids including benzoic, phenylacetic and phenylpropionic acids, may be oxidized with hydrogen peroxide so as to yield among other products *acetaldehyde*, acetic acid and carbon dioxide. Since acetaldehyde according to Friedmann may be converted in the liver into aceto-acetic acid which yields acetone, it is possible to correlate these

[1] If oxidation of phenylserine or the phenylglyceric acids had taken place in the α-position, mandelic acid, C_6H_5. CHOH COOH, would have been found in the urine.

[2] This *Journal*, v, p. 183.

[3] *Arch. f. exp. Pathol. u. Pharm.*, lix, p. 273.

[4] This *Journal*, iii, p. 419.

facts with Embden, Salomon and Schmidt's[1] observation of the formation of acetone during the perfusion of surviving livers with blood containing phenylalanine or tyrosine.

EXPERIMENTAL.

The Fate of Phenylalanine. Inactive phenylalanine, 8.0 grams, was dissolved in 300 cc. of warm salt solution and slowly injected into the femoral vein of a cat weighing about 4.0 kilos. The time of injection was approximately 1½ hours. At the close of the experiment the animal was replaced in its cage and the urine passed during the injection and also during the next ten hours was carefully collected. On standing, a small quantity of crystalline substance was deposited round the edges of the liquid. On examination the substance proved to be α-ureido-β-phenylpropionic acid and was obtained later in larger quantity. The fact that a small quantity crystallized out directly from the urine is of importance, since it removes the possible objection that the substance resulted from the analytical procedures. The urine was examined with negative results for phenyl-α-oxypropionic acid, phenaceturic acid, homogentisic acid and tyrosin. On concentrating the urine a large quantity of crystals was obtained, but they proved to be largely composed of unchanged inactive phenylalanine. In all about 2.0 grams of phenylalanine were recovered. The urea derivative was separated from the amino-acid by extraction of the acidified urine with hot ethyl acetate in a continuous extractor. The ethyl acetate extract was purified by distillation in steam and treatment with charcoal in the usual manner and gave on concentration 1.1 grams of crystals of α-uramido-β-propionic acid. The substance crystallizes in small, thick prisms and melts at 188–190° with decomposition. If the substance is heated rapidly, as high a melting point as 190–191° is obtained. When mixed with the synthetic substance (see below) the melting point was unchanged. The substance was optically inactive.

Analysis:

0.1189 gram gave NH_3 = 0.01596 gm.; N = 13.42 per cent N
$C_{10}H_{12}O_3N_2$ requires 13.46 per cent N

[1] Hofmeister's *Beiträge*, viii, p. 148.

Synthesis of α-uramido-β-phenylpropionic acid. By evaporating on the water bath an aqueous solution of equal weights of phenylalanine and potassium cyanate and then adding a slight excess of dilute hydrochloric acid to the residue, α-uramido-β-phenylpropionic acid is readily obtained. It is purified by recrystallization from water and melts at 188–190°. The yield of pure substance is about 75 per cent of the theoretical amount.

Analysis:

0.1552 gram gave NH_3 = 0.0210 gm, : N = 13.53 per cent
$C_{10}H_{12}O_3N_2$ requires 13.46 per cent

Fate of Phenyl-β-alanine. Phenyl-β-alanine was first obtained by Posen[1] by the action of aqueous ammonia upon phenyl-β-bromopropionic acid. It was found difficult to obtain a product free from ammonium cinnamate by following Posen's directions which were modified as follows: Phenyl-β-bromopropionic acid was added in small portions to seven times its weight of aqueous ammonia (sp. gr., 0.9) cooled in a mixture of ice and salt. After about an hour the solution was diluted somewhat and filtered to remove the bulk of the styrol, and evaporated slowly on the water bath until the larger part of the excess of ammonia was removed. The solution was made just acid to congo red with sulphuric acid and the cinnamic acid removed by four extractions with ether. The aqueous portion was rendered slightly alkaline with ammonia, and on concentration gave crystals of phenyl-β-alanine, which were carefully washed with water to remove ammonium salts. The yield of pure amino-acid is about 25 per cent of the theoretical amount. The substance crystallized from water in large, flat, colorless platelets and melted at 122–123°. Posen gives 121° as the melting point.

Phenyl-β-alanine prepared as above was given subcutaneously in two-gram doses to a cat and to a small dog of about 7.5 kilos. In the case of the cat a small quantity of unchanged acid was recovered from the urine, but otherwise the results were identical. The urine was analyzed by the methods previously employed. Phenyl-β-oxypropionic acid (0.7 gram) was obtained in crystalline form and melted at 92°. The acid was decidedly laevo-rotatory,

[1] Liebig's *Annalen*, cxcv, p. 144.

and was identified by all the usual tests, including conversion into cinnamic acid, m.p. 132°, and oxidation to acetophenone with chromic acid. Acetophenone (8 milligrams) was detected in the urine and gave the characteristic nitroprusside reaction. Hippuric acid (0.7 gram) was obtained in the form of good crystals, m.p. 186–187°. No indications of the presence of cinnamoylglycocoll were obtained.

Fate of Phenylserine. Phenylserine was prepared according to Erlenmeyer and Früstück's method[1] by the action of acetic acid upon the product obtained by the condensation of 2 mols. of benzaldehyde with 1 mol. of glycocoll. 1.5 grams dissolved in water containing a trace of sodium bicarbonate were given subcutaneously to a cat weighing 3.9 kilos. The urine was examined according to the usual methods and 0.9 gram of hippuric acid crystals was obtained. No other substances were detected. The experiment was repeated with identical results.

Fate of the Phenylglyceric Acids. One of the two isomeric modifications of phenylglyceric acid (m. p. 141–142°) was obtained by the oxidation of cinnamic acid in cold alkaline solution with potassium permanganate according to Fittig and Ruer's directions.[2] The other modification was obtained by the action of alkali upon phenyl-α-chloro-β-oxypropionic acid. No difference could be detected in the behavior in the body of the two acids and, although some unchanged acid was excreted in each case, the recovered acid failed to show any sign of optical activity. In two experiments in which 2.0 grams of each acid were given to a dog weighing 8.8 kilos, 0.25–0.3 gram of hippuric acid was recovered from the urine, together with 1.0–1.2 grams of unchanged phenylglyceric acid. The unchanged phenylglyceric acid was obtained by extracting the syrupy mother-liquor from the hippuric acid crystals with ether.

When similar experiments were made with cats, no hippuric acid was obtained, but about 65 per cent of the acids administered was recovered. It is clear that the phenylglyceric acids are comparatively resistant to oxidation in the animal body.

Fate of Phenylacetaldehyde. This substance was obtained from

[1] Liebig's *Annalen*, cclxxxiv, p. 45.

[2] *Ibid.*, cclxviii, p. 27.

phenyl-β-chloro-α-oxypropionic acid by Erlenmeyer and Lipp's method.[1] Three grams of the aldehyde were dissolved in dilute alcohol and given subcutaneously to a large dog (19 kilos). Absorption of the aldehyde appeared to be slow. 0.7 gram of phenaceturic acid was obtained from the urine. In another experiment about 1.0 gram of phenaceturic acid was obtained after 4.0 grams of the aldehyde had been administered.

[1] Liebig's *Annalen*, ccxix, p. 182.

THE NUCLEIN FERMENTS OF YEAST.

By M. N. STRAUGHN and WALTER JONES.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

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In the year 1874 Schützenberger[1] made an investigation of the products of the self-digestion of beer-yeast and found among other substances xanthin, hypoxanthin and guanin. He looked upon these compounds not as preformed constituents of the yeast, but as products of ferment action and exercised great care that putrefactive changes be excluded. But the chemistry of the nucleic acids was so little understood at this time that Schützenberger was not in a position to draw correct conclusions from the results which he obtained. Moreover, the methods of separating the xanthin bases from one another were so imperfect that Schützenberger's findings cannot be accepted as conclusive. He found both guanin and xanthin. If guanase is present in the yeast one would scarcely expect to find guanin among the products of self-digestion, while in the absence of guanase it is equally difficult to account for the presence of xanthin unless the substance was produced from guanin by a laboratory process.

Eleven years later Lehmann[2] undertook the same investigation, but under much more favorable conditions: for the nucleic acids and their decomposition products had been closely studied and the view had been clearly proposed that the xanthin bases which occur among the autolytic products of gland extracts are produced from nucleic acids by the action of ferments present in the tissues. Lehmann made a quantitative estimation of the xanthin bases present after the action of boiling mineral acid, first upon aqueous extract of fresh yeast, second upon extract of yeast that had stood at the room-temperature for 24 hours,

[1] *Bull. de la soc. chim. de Paris*, p. 194. 1874.
[2] *Zeitschr. f. physiol Chem.*, ix, p. 563, 1885.

and third upon yeast extract that had been kept for an equal time at the body-temperature. From the results thus obtained he concluded that in the course of the digestion the hypoxanthin decreases while the sum of the guanin and xanthin increases. The conversion of hypoxanthin into xanthin would only require the presence of an oxidizing ferment, xanthoöxidase, whose occurrence in gland extracts has been so frequently demonstrated that its presence in this connection might readily be granted; but we propose to show that the presence of xanthoöxidase in the yeast cannot be demonstrated. So far as concerns the increase of guanin with a corresponding decrease of hypoxanthin, it should be noted that the transformation thus suggested would involve at some stage the insertion of an amido group which would be contrary to all modern work, since the withdrawal of amido groups from the purin ring is a well-known function of the intracellular ferments.

If, however, we ignore Lehmann's conclusion and examine his results in the light of modern discovery they will be found to admit of an interpretation which is in agreement with our present notions of nuclein fermentation and which we shall confirm in this communication. In the following duplicate tables taken from Lehmann's article the figures of the first column were obtained from fresh yeast extract, those of the second and third from extracts that had stood for 24 hours at the room-temperature and at the body-temperature respectively.

	I	II	III
Hypoxanthin	0.2110	0.2188	0.0212
Guanin	0.0902	0.0140	0.0123
Xanthin	none	0.0731	0.1210
Hypoxanthin	0.1600	0.1736	0.0239
Guanin	none	traces	none
Xanthin	0.0383	0.0509	0.1337

The methods of separation which were at Lehmann's disposal (and which he does not describe) must have been very crude and some of the perfectly apparent confusion of his table is to be ascribed to this cause; but his figures nevertheless show that as

digestion proceeds the guanin decreases and disappears, while the xanthin correspondingly increases. This transformation may be accounted for by the action of guanase, whose presence in the yeast we propose to demonstrate.

Lehmann's work is important as the first investigation which indicated the presence in cells of ferments capable of bringing about an alteration of xanthin bases, but his results in common with those of all his predecessors contained a fatal though unavoidable defect. Kossel's discovery of adenin had not yet been made, so that the important rôle which this base plays in autolysis could not be taken into consideration by the earlier investigators.

The more recent work of Kutscher[1] would indicate that neither of the disamidifying ferments is present in the yeast, since he found among the products of self-digestion both guanin and adenin, but was unable to prove the presence of xanthin.

Finally, Shiga[2] studied the action of yeast on added guanin. As digestion proceeded, he noted a decrease in the guanin and an increase of xanthin; but at the same time there occurred a marked increase in adenin, a result which Shiga pronounces most unexpected and difficult to explain.

In the following pages it is our purpose to show that the yeast rapidly converts guanin into xanthin (and, therefore, contains the ferment guanase), but is not capable of bringing about the analagous transformation of adenin into hypoxanthin nor of oxidizing hypoxanthin to xanthin; hence adenase and xanthooxidase are absent. The yeast is peculiarly well adapted to studies of this kind. Aqueous infusions can be obtained by the use of the centrifuge which are almost as clear as water, while the small amount of blood pigment, unavoidable in the case of most glands, does not produce its usual annoyance here. The analytical work, therefore, yields clean results and furnishes a most conclusive instance of the occurrence of guanase in the absence of the other purin ferments.

I. Xanthoöxidase. The functions of this ferment are the conversion of hypoxanthin to xanthin and of xanthin to uric acid, and its presence in a tissue is not likely to escape observation.

[1] *Zeitschr. f. physiol. Chem.*, xxxii, p. 59.

[2] *Ibid.*, xlii, p. 502.

In the first place the oxidation of hypoxanthin proceeds with great ease and there is always enough available oxygen in gland extracts to effect the transformation, so that one is not likely to find even a trace of hypoxanthin in a digested tissue extract when xanthoöxidase is present. Again, a small amount of uric acid is always formed in the digestion of glands which in any manner produce xanthin in the presence of xanthoöxidase, even though no air is passed into the material. This uric acid, even when present only in traces, will be noted in making the color test for xanthin; for in such a case the yellow spot ordinarily obtained by the evaporation of xanthin with nitric acid will be rings differing from one another in composition and showing different colors, viz., the bright yellow due to xanthin and the pink due to uric acid. When such a deposit is moistened with caustic soda the yellow and pink change to blood red and violet respectively, and the distinction is such that a small fraction of a milligram of uric acid can be detected in the presence of ten times the weight of xanthin. A number of experiments made with yeast produced specimens of xanthin which showed no trace of uric acid in the color test as described; but to settle the point beyond doubt the following experiment was undertaken.

Three hundred grams of compressed yeast were placed in a closed vessel with a liter of distilled water and 6 cc. of chloroform, and the materials allowed to remain at the room-temperature for 15 hours with frequent and violent agitation. The starch and other insoluble materials were then sharply removed with the centrifuge. To 750 cc. of this aqueous extract were added 400 mg. of hypoxanthin dissolved by suspending in warm water and adding the amount of caustic soda which just sufficed for the complete solution of the base. The materials were then kept at 38° for four days and during about half the time a slow stream of air from a wash bottle containing chloroform was passed through the digesting fluid. At the end of the digestion a drop of acetic acid was added and after heating to the boiling point the hot fluid was filtered from the coagulum. The filtrate was treated with 10 cc. of dilute sulphuric acid, evaporated to about 150 cc. and boiled for half an hour to decompose any trace of proteid that may have been present. The liquid was then made strongly alkaline with ammonia, the bases were precipitated

with an ammoniacal solution of silver nitrate, and after decomposing the silver compounds with hydrochloric acid the filtrate from silver chloride was evaporated for the expulsion of the excess of acid. Care was taken, however, to avoid heating the material even for a moment after it had become dry, and the same precaution was observed in the evaporation of the water from the moistened residue. The dry product thus obtained was found quite easily soluble in water at 40°, leaving only a trace of pigmented granular material which responded to the color reaction for xanthin without giving a suggestion of the colors produced by uric acid (evidently a small amount of xanthin from the nucleic acid of the yeast extract). The filtrate which contained the great bulk of the material under examination was made decidedly alkaline with ammonia. The precipitated phosphates were filtered off and examined for guanin, but with negative results, while the filtrate was treated with picric acid. After filtering off a small precipitate thus produced (evidently a small amount of adenin picrate due to the nucleic acid in the yeast extract), the picric acid was removed from the solution with sulphuric acid and ether and the hypoxanthin was precipitated with ammonia and silver nitrate and converted into its characteristic nitrate by the usual method. The crystals of hypoxanthin nitrate thus obtained showed the whetstone forms, and upon evaporation on a porcelain surface with a drop of nitric acid, left only a pale lemon-yellow spot whose color was scarcely intensified by the addition of a drop of caustic soda. The nitrate was converted into the free base, which was analyzed.

0.1381 gm. required 7.78 cc. of standard sulphuric acid (1 cc. = .00724 gm. N).

	Required for hypoxanthin:	Found:
N	41.2 per cent.	40.7 per cent.

Hypoxanthin introduced .....400 mg.
Hypoxanthin nitrate recovered.....540 mg. (90 per cent).

The results described show that the yeast does not contain xanthooxidase, and indicate that it contains guanase but not adenase.

II. Guanase. The methods employed in searching for this ferment were essentially those which we have frequently described in connection with similar work upon animal glands.

As the presence of guanase was indicated in the previous experiment, we introduced a comparatively large amount of guanin in order that the ferment might have a relatively great amount of work to do and thus give an undoubted proof of its presence. To 500 cc. of aqueous extract of yeast were added 600 mg. of guanin sulphate dissolved in the smallest possible amount of caustic soda, and the digestion of the material was allowed to proceed at 37° for 4 days. At the end of this time the products were removed and examined for xanthin bases by the method already described. As there was a possibility of finding guanin the final residue obtained after evaporation of the acid filtrate from silver chloride was treated with water at 40° and, without filtering, the fluid was made markedly alkaline with ammonia. The precipitated phosphates were treated with hot 1 per cent caustic soda for the solution of guanin and the alkaline fluid faintly acidified with acetic acid. Not the slightest turbidity occurred, showing the total absence of guanin. The alkaline filtrate from phosphates was treated with silver nitrate in ammonia, the precipitated silver compounds were decomposed with hydrochloric acid, and the filtrate from silver chloride was evaporated to expel the excess of acid. The residue was digested with water at 40°, but the great bulk of the material remained as heavy yellow grains. The fluid was filtered off and the insoluble substance (which could be only xanthin) was washed first with cold water, then with hot water acidified with acetic acid and finally with alcohol and ether. The dry product weighed 382 milligrams (equivalent to 91 per cent of introduced guanin) and gave an intense color reaction for xanthin. This material was joined to other specimens of xanthin similarly obtained by the action of yeast extract on guanin and purified by conversion into the nitrate; from the pure nitrate the free base was again prepared and analyzed.

The filtrate from xanthin gave a precipitate with picric acid, indicating the presence of adenin.

The observation that guanin when digested with an organ extract partly or entirely disappears and that an equivalent amount of xanthin can be isolated from the products cannot be accepted as a proof or even an indication of the presence of guanase unless the observer has convinced himself by check tests or in other ways that his chemical procedure is in no way responsible

for the supposed transformation. This precaution is specially to the point where one finds traces of alteration products. For instance, one uses in a digestion a relatively large amount of guanin (500 milligrams), which was prepared originally from a gland and was associated with xanthin from which it has *presumably* been purified. At the end of the digestion the finding of 10–20 milligrams of xanthin among the products is no indication whatever that guanase is present even in traces.

The great source of danger, however, lies in the use of a nitrate as a reagent in the isolation of the products. The bases are precipitated with a solution of silver nitrate in ammonia, and should any of this reagent escape removal in washing the gelatinous precipitate, the succeeding decomposition of the silver compounds with an excess of hydrochloric acid would bring about ideal conditions for the conversion of amidopurins into oxypurins, and thus produce misleading results. In the work here described as well as in all previous work we have been constantly on our guard against errors of this kind. Upon occasion we have verified our results by experiments in which an ammoniacal solution of silver sulphate was employed instead of the nitrate. Moreover, the frequently proved absence of the disamidifying ferments shows that their assumed presence in other connections is not a matter of chemical manipulation on our part. All possibility of confusion, however, may be avoided and at the same time the convenient form of reagent (silver nitrate) may be employed if the thorough washing of the silver precipitate is effected by piercing the filter, spurting the precipitate into a flask with a jet of boiling water and, after violent agitation and cooling, filtering on to a fresh filter. After this has been repeated several times the filtrate is so far free from ammonia that it will not turn sensitive red litmus and presumably all soluble constituents have been removed.

Although we have not the remotest idea that the conversion of guanin into xanthin above noted has any other cause than the presence of a ferment in the yeast, yet the following experiment will demonstrate how the alteration product can be gained possession of so quickly that all doubt as to its origin is excluded. To 750 cc. of yeast extract were added 500 milligrams of guanin hydrochlorate, and the digestion carried on as stated. The

product was treated with a drop of acetic acid, heated to boiling and the coagulum filtered off. The filtrate was strongly acidified with sulphuric acid and after evaporation to about 200 cc. was allowed to stand over-night. A deposition of a large amount of pale-yellow crusts had occurred. This material, which, owing to the high acidity and great dilution of the fluid, could not be guanin, was removed and washed on the centrifuge with water and and finally dried with alcohol and ether. It weighed 285 milligrams (this amount being equivalent to 81 per cent of the guanin employed). Tests with small quantities of material showed that the substance was soluble with great ease in 0.5 per cent ammonia and insoluble in a great excess of 2 per cent sulphuric acid. It gave an intense color reaction for xanthin, and its solution in ammonia produced a heavy, gelatinous precipitate with an ammoniacal solution of silver nitrate. The bulk of this material (which could be no xanthin base but xanthin) was purified by conversion into the nitrate and from the nitrate the pure base was prepared and analyzed.

0.1266 gm. required 6.48 cc. standard sulphuric acid (1 cc. = 0.00724 gm. N).

	Required for xanthin:	Found:
N.....	36.8 per cent.	37.0 per cent.

The impure xanthin obtained in three experiments after the addition of guanin to the yeast weighed 1.055 grams. This was purified through the nitrate and 975 milligrams of pure xanthin obtained, which was analyzed.

0.2024 gm. required 10.2 cc. of standard sulphuric acid (1 cc. = 0.00724 gm. N).

	Required for xanthin:	Found:
N.....	36.8 per cent.	36.5 per cent.

Thus the yeast is capable of transforming relatively large quantities of guanin into xanthin, i. e., it contains guanase.

III. Adenase. Results already described point distinctly to the failure of this ferment in the yeast. In the experiment with hypoxanthin as well as in a number with guanin, besides the product found in large amount and due to the added material, there

was always obtained a precipitate with picric acid at the point in the analytical procedure where we would expect to find adenin. However, a number of experiments were undertaken for the purpose of dealing specially with the question of adenase. The arrangement of the digestion and the process employed for the isolation of the products have in the main already been described, so that a repetition is not necessary here. The results of two experiments are as follows:

1 *Yeast extract 500 cc.*

Adenin sulphate added	500 mg.	
Equivalent adenin	333 "	
Adenin picrate obtained	915 "	
Equivalent adenin	320 "	or 96 per cent.

2 *Yeast extract 500 cc.*

Adenin sulphate added	500 mg.	
Equivalent adenin	333 "	
Adenin picrate obtained	821 "	
Equivalent adenin	287 "	or 86 per cent.

The *presence* of a ferment is best proven by showing that a relatively large amount of introduced material can be transformed in a short time; but to prove the *absence* of a ferment it should be shown that small quantities of material are not altered. The least possible work that can be imposed upon a nuclein ferment is in the autodigestion of aqueous extracts of the tissues, for in this case the ferment has only to take care of the small amount of base formed from the nucleic acid of the tissue. Accordingly 2000 cc. of aqueous extract of yeast (prepared as described) were submitted to self-digestion at 37° for 4 days, and the products examined by the usual method. There were obtained 132 milligrams of xanthin and 345 milligrams of adenin picrate (equivalent to 121 milligrams of adenin). Guanin could not be found.

Having proven that the yeast contains no adenase, since the extract cannot convert the small amount of adenin formed from its own nucleic acid, we proceed to the consideration of a phenomenon that we have had occasion to observe in connection both with yeast and with certain animal glands. In examining the products formed by the action of yeast on adenin it was observed that after the precipitation of the unaltered adenin with picric acid, the filtrate from adenin picrate produced a small precipitate

with silver nitrate and ammonia. In attempting to account for this we at first supposed that the specimen of adenin sulphate employed in the experiments might possibly have contained a trace of hypoxanthin as an impurity; but it was found that this same specimen of adenin sulphate in aqueous solution was completely precipitated by picric acid, so that the filtrate from the adenin picrate gave no precipitate with silver nitrate and ammonia. Moreover, this same doubtful trace of hypoxanthin was found among the products of the self-digestion of yeast, in which case, of course, it could not be accounted an impurity. The formation of a silver precipitate in a filtrate from adenin picrate might be attributed to the slight solubility of adenin picrate itself, and this is the conclusion which we reached in connection with our first observations; but an accumulation of instances, supplemented by tests made for the exclusion of various explanations has led us to the conclusion that certain tissues (perhaps all glands) contain a trace of hypoxanthin which has no direct connection with the ferments of the nuclein metabolism, and which surely does not owe its presence to the decomposition of adenin by adenase since this ferment is not present in the tissues referred to. The proof of a small amount of preformed hypoxanthin in aqueous tissue extracts is not a simple matter. If the tissue in question contains adenase the finding of hypoxanthin would be attributed to the action of the ferment on adenin. Again, if the tissue contains xanthoöxidase a trace of preformed hypoxanthin would be quickly oxidized to xanthin or uric acid; so that the proof of preformed hypoxanthin depends upon experiments with tissues that contain neither of these two ferments. Such a tissue is the human spleen. In a number of experiments recently made with this organ,[1] it was observed that a small amount of a purin base, precipitable by silver nitrate and ammonia, was present in the filtrate from adenin picrate. As the amount of the base was small, as a number of hypotheses could explain its presence, and as it has no bearing upon the point then under consideration (the presence or absence of adenase in the tissue), the substance was neglected. When the same phenomenon was repeatedly observed in our experiments with yeast, we took occasion to collect

[1] Winternitz and Jones: Forthcoming number of *Zeitschr. f. physiol. Chem.*

enough material to satisfy ourselves of its identity and in its isolation we employed such methods and made such check tests as to convince ourselves that the substance in hypoxanthin and that its presence is due neither to adenase in the yeast extract nor to a decomposition of adenin by the methods of isolation employed.

This matter calls to mind the more abundant occurrence of hypoxanthin in muscle, where the great amount of the base is out of all proportion to the amount of nuclear material, and where xanthin (or its equivalent, guanin), the constant physiological companion of hypoxanthin, is to be found only in traces. The work of Schittenhelm[1] on the nuclein ferments of muscle goes to show that the two disamidifying ferments are plentifully present in this tissue (both of man and animals), so that the occurrence of adenase would account for hypoxanthin as a product of nuclein metabolism. But an investigation now being carried on in this laboratory by V. N. Leonard proves very conclusively that there is no ferment in dog's muscle capable of causing the conversion of adenin into hypoxanthin. We therefore feel justified in concluding that a small amount of preformed hypoxanthin exists in various organs, which, as well as the more abundant occurrence of the base in muscle, has no direct connection with the ferments of the nuclein metabolism.

[1] Schittenhelm and Schmid: *Zeitschr. f. exp. Pathol. u. Ther.*, iv, p. 427.

FURTHER STUDIES ON THE USE OF THE FERMENTATION TUBE IN INTESTINAL BACTERIOLOGY.

By A. I. KENDALL

(Fellow of the Rockefeller Institute for Medical Research).

(*From the Laboratory of Dr. C. A. Herter, New York.*)

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In a previous communication[1] attention was called to useful information derivable from the study of the gas volume and Gram-stained sediments in fermentation tubes inoculated with feces.

Mention was made of the fact that the vast majority of bacterial types composing the fecal flora in health and disease can develop rapidly in these tubes during the first eighteen hours of incubation, and that the resulting growths are, broadly speaking, to be regarded as indices of the *relative viability* of the various components of this fecal flora as it existed at the time the tubes were inoculated. During this incubation period of eighteen hours, the nutrient material derived from the fecal suspension (which was added with the bacteria at the time of inoculation) suffices to enrich the artificial medium in the fermentation tube to such an extent that even those bacteria which grow poorly or even not at all in the artificial media alone (e. g., *B. bifidus*) proliferate rapidly under these special conditions. At the same time the mixed products of bacterial activity (which doubtless are present in relatively concentrated form in the feces) are so considerably diluted by the media in the fermentation tubes that the various types of bacteria, freed from the inhibitory action of these substances, are enabled to vegetate rapidly from the beginning of incubation in an unusually favorable environment.

It was shown, furthermore, that actively vegetating bacterial cells, which are found in the sediments of these fermentation tubes, are much more distinctive, morphologically, than is the

[1] Herter and Kendall: This *Journal*, v, p. 283, 1908.

case in the stools, where these organisms are subjected on the one hand to the deleterious effects of partial desiccation due to withdrawal of water from the fecal masses in the large intestine and on the other to the various products of bacterial activity which become concentrated in the feces.

The result of this exposure to unfavorable conditions is manifested by a depression of bacterial vegetative activity. This modifies the morphology of the fecal flora both by causing a diminution in size in many varieties and well-marked changes in the cellular appearances and staining reactions.

In very young children, where the intestinal flora is relatively simple, and composed chiefly of a few dominant types, the study of the phenomena developed in the fermentation tubes (gas-volume and character of the sediment) furnish particularly valuable indications relative to the bacterial conditions obtaining in the intestinal tract.

Escherich,[1] Tissier,[2] Szegö[3] and others have studied the distribution of bacteria in the alimentary tract and have noticed that in perfectly normal enteric conditions the upper levels are inhabited mainly by facultative aërobic bacteria, while the lower portions of the colon are dominated by anaërobic bacteria. (Escherich[4] referred to the anaërobic bacilli of the bifidus type as "blue colored colon bacilli").

Repeated examinations of the flora from healthy intestinal contents, both culturally and with the aid of the fermentation tubes, have confirmed these observations. Marked deviations from this norm, most expeditiously detected by the use of fermentation tubes, are readily discernible, and although in the light of the information at present available it is not possible to fully interpret the finer and more subtle phases of these variations, the more general and distinctive changes to which must be attached the most significance can be confidently outlined.

The following observations bear upon certain seeming discrepancies which often are met with in the routine use of the fermenta-

[1] Escherich: *Darmbakterien des Säuglings*, Stuttgart, 1886.

[2] Tissier: *Recherches sur la flore intestinale du nourrisson*, Paris, 1900.

[3] Szegö: Die Darmmikroben der Säuglinge und Kinder, *Arch. f. Kinderheilk.*, xxii, 1897.

[4] Escherich: *loc. cit.*; also *Jahrb. f. Kinderheilk.*, xlix, p. 138, 1899.

tion tubes in connection with the study of the fecal bacteria. These are commonly of two types:

A. Abnormal gas volume in one or two of the three commonly used sugars;

B. Apparent incompatibility between the gas volume and the character of the bacteria observed in the Gram-stained sediment of the tube.

A. *Abnormal Gas Volume.* Generally speaking the amount of gas produced respectively in dextrose, lactose and saccharose is about the same under normal conditions; that is, one usually finds that the mixed fecal bacteria liberate about the same percentage of gas from each of these carbohydrates during the first eighteen hours of incubation at 37° C.

(If fermentation tubes of different sizes are used for the different sugars, the larger tubes, irrespective of the carbohydrates contained in them, will usually contain greater volumes of gas than will the smaller tubes. This difference is more apparent than real, however. Upon comparing the length of the gas column with the total length of the closed arm of the fermentation tube one will find that in each instance the *total per cent* of gas is very nearly the same irrespective of moderate fluctuations in the relative size of the tubes used for this purpose.)

While the above statements are true for the majority of normal stools, the average of an extensive series of observations shows that a slightly greater volume of gas is actually produced in dextrose than in either lactose or saccharose: the proportion is about 10 to 9, comparing dextrose and lactose directly. Saccharose and lactose, on the contrary, run very nearly the same in this respect.

One fact stands out rather clearly: Lactose is less easily attacked in general by facultative intestinal bacteria and invading organisms than by the obligate intestinal bacteria. Many of the foreign bacteria produce *alkali* in this carbohydrate, while they generate *acid* in dextrose and saccharose under the same conditions, indicating very clearly that in the former case the organism is deriving its oxygen from the air, or at least is unable to obtain it directly from lactose, while in dextrose and saccharose the oxygen may be derived directly through the combustion of the carbohydrates, which are thus shown to be immediately assimilable.

This lack of assimilative power for lactose is even more strikingly illustrated by the growth of certain proteolytic bacteria of the subtilis type in the fermentation tubes. In lactose frequently there is no growth in the closed arm (provided the lactose is not hydrolyzed) and there is formed a heavy pellicle upon the surface of the open arm, together with a moderate turbidity in the bulb. This is a strong indication that the bacillus is obtaining its oxygen from the air rather than from the lactose. In dextrose and saccharose, on the contrary, there is a well-marked turbidity in the closed arm and there is no pellicle upon the free surface of the bulb, at least not for forty-eight hours or more. The reaction is usually slightly acid in these sugars, alkaline in lactose.

Having shown that lactose (a carbohydrate peculiar to animals) is in general less readily attacked by facultative and foreign races of bacteria than are dextrose and saccharose (of vegetable origin), and that the obligate intestinal organisms are able to utilize lactose as well as other sugars in their dietary, the significance of abnormal gas volumes becomes more definite.

Broadly speaking the gas volume produced by mixed fecal bacteria depends upon the relative activities of three distinct types of bacteria: the gas-producing type (aërogenic), the acidogenic type (acidophile and coccal forms chiefly), and those bacteria which produce alkali.

The gas-producing bacteria, when they are dominant in the feces (or in pure culture), as a rule produce a volume of gas equal to about 25 or 35 per cent of the total length of the closed arm of the tube. Experiments carried out by the writer have demonstrated that this gas volume may be increased to a considerable degree when these gas-producing bacteria are growing symbiotically with certain strains of alkali-producing bacteria. The latter appear to neutralize the acid produced by the former to a considerable extent, and the period of gas formation is consequently lengthened by this partial removal of acid which impedes the vegetative growth of these organisms.

Not all alkali-producing bacteria can grow symbiotically with the aërogenic bacteria, however. *B. infantilis*,[1] for example, inhibits the growth of certain of the most common gas-producing

[1] Kendall: This *Journal*, v, p. 419, 1909.

bacilli (*B. coli* and *B. aërogenes*) probably through the production of unfavorable metabolic products. This is true in dextrose and in saccharose (provided the strain of colon bacillus is one that produces gas in the latter carbohydrate; it will be remembered that certain strains cannot ferment this carbohydrate and, naturally, cannot be considered in this discussion), but the reverse is true in lactose. In lactose fermentation tubes inoculated with *B. coli* and *B. infantilis*, the gas volume remains essentially the same as that of *B. coli* alone, while *B. infantilis* grows very slowly in this medium, eventually producing a pellicle, but no turbidity. This organism also produces alkali in lactose when it is grown in pure culture.

Those bacteria which produce acid, on the contrary, and no gas (and we are familiar with three well-defined types, the coccal forms, including the intestinal streptococci and micrococci, the anaërobic bifid forms, and the facultative aërobic *B. acidophilus* forms) react in a totally different manner with the aërogenic bacilli. These acidogenic organisms augment the amount of acid produced by the aërogenic bacilli of the coli-aërogenes type, and inasmuch as they frequently proliferate rapidly, they inhibit the growth of the gas-forming bacteria. Evidences of this inhibition are frequently seen almost at the beginning of incubation, and a mixed fecal flora which contains these acidogenic forms, either in large numbers, or as varieties which are in an active state of vegetative reproduction, frequently does not generate gas, or at most only small amounts, much less than normal. Stools not infrequently contain acidogenic bacteria of the acidophile type in large numbers, or in a state of active vegetative reproduction. Such stools are usually relatively desiccated, and as a rule specify a relatively long sojourn in the lower portions of the colon. The coli-aërogenes type of bacilli on the contrary find their most suitable environment in the region of the ileocecal valve, the cecum and at particular levels of the small intestine. Hence, when they are carried to the rectal region, they are exposed not only to the products of their own activity produced in the higher levels of the intestinal tract, but also to the products of metabolism of the acidogens in the lower colon as well as to partial desiccation associated with the withdrawal of water from the fecal mass prior to defecation.

The result of this exposure to an unfavorable environment is a depression of the vitality of the colon-aërogenes bacilli.

This depression of vitality is manifested by the inability of these bacteria to grow with their characteristic luxuriance when they are introduced as a part of the fecal flora into fermentation tubes as outlined above. That this lack of development is due to a depression of vitality rather than attributable to actual death is evidenced by the fact that one may easily recover strains of these bacilli if they are grown in non-saccharine media (where the acidogens no longer can produce acid) or by their isolation from plate cultures.

The result of the inability of the colon bacilli to grow in the fermentation tubes under these conditions is a diminution in the amount or even an absence of gas in the closed arm.

To recapitulate: The gas volume is, broadly speaking, the resultant of the combined activities of three types of bacterial action—the aërogenic (brought about usually by *B. coli* and *B. aërogenes*, commonly), the acidogenic (*B. bifidus* and various strains of *B. acidophilus* as well as the intestinal cocci) and the alkali-producing forms.

The gas is chiefly produced by organisms of the colon type. The amount of gas evolved when these bacteria are dominant is about the same as that produced by pure cultures of these organisms.[1]

The limitation of this gas volume is frequently referable to decreased vegetative activity of the colon-aërogenes bacteria rather than to exhaustion of the food supply, as may be shown by diluting cultures in which the evolution of gas has ceased, with sterile water, or, better, sterile physiological salt solution. When this is done a fresh evolution of gas usually will take place.

This fact may be even more clearly brought out by the use of media containing calcium carbonate in addition to the carbohydrate. Protocols of a few typical experiments with the bacteria of normal babies' stools are tabulated. Control experiments to determine the effect of lime on the various types of bac-

[1] Mention has been made of the increased gas volume that is not infrequently noticed when the gas bacillus (*B. perfringens* or *Bact. Welchii*) is present in unusual numbers (Herter and Kendall, *loc. cit.*). In unusual instances *B. vulgaris* (*Proteus vulgarus*) may increase the amount of gas.

teria failed to reveal any selective action favorable to the growth of the aërogenic bacteria, and it is therefore very probable that the neutralization of the acids formed by the colon bacilli and by the acidogenic types of organisms is the most potent factor associated with the increased gas production in carbohydrate-calcium carbonate media. The parallel horizontal columns indicate the amounts of gas (in millimeters) produced respectively in dextrose, lactose and saccharose, both with and without calcium carbonate, using for this purpose fermentation tubes of the regulation type, and the mixed fecal flora of healthy babies.

WITHOUT $CaCO_3$			WITH $CaCO_3$		
Dextrose	Lactose	Saccharose	Dextrose	Lactose	Saccharose
20	28	28	51	70	65
15	16	17	60	80	72
21	26	21	60	64	55
22	22	23	73	80	63
2	0	3	32	37	35
17	16	16	76	79	74
15	16	15	55	72	60
7	12	8	45	65	48

Not infrequently one sees a decided augmentation of the gas volume in either dextrose, saccharose or both. It is less common in lactose. One frequently finds in such cases a decided increase in the number of alkali-producing bacteria, particularly of the subtiloid type, many strains of which are capable of growing symbiotically with *B. coli*.

One other instance in which the gas volume in dextrose may be markedly increased deserves mention. The writer has found five cases of this kind among young rachitic babies. There was three times as much gas in dextrose as in either lactose or saccharose in three of these cases. Subsequent investigation showed that there were fairly large numbers of typical paratyphoid bacilli in the feces of these children. These bacteria, it is well known, ferment dextrose with the production of gas, but are unable to form gas in either lactose or saccharose. Cultural experiments indicated that *B. coli* was replaced to a considerable extent by

these paratyphoid bacilli. The presence of paratyphoid bacilli was quite unsuspected in these cases and it was only after the first case had been worked out that a similar possibility was suspected in the other four cases. Investigation showed that this supposition was correct.

B. *Apparent Incompatibility between Gas Volume and the Character of the Bacteria in the Gram-stained Sediments.* This phenomenon, most frequently associated with a very small gas production, and the presence of comparatively large numbers of Gram-negative bacilli of the colon-aërogenes types in the Gram-stained sediment, is difficult to explain.

Cultural examination of the bacteria in such fermentation tubes presenting such apparent incompatibilities between gas volume and character of bacteria always show that viable colon bacilli (or *Bact. aërogenes*) are present in at least moderate numbers, and that they may be cultivated without difficulty as soon as they are removed from the direct influence of products of the vital activity of the acidophile types. The latter, it will be remembered, are noteworthy chiefly because they can grow in the presence of acid of sufficient strength to inhibit the vegetative reproduction of other types of bacteria.

The colon bacilli associated with these acidophile bacteria are perfectly typical in every respect, culturally and morphologically, stain in a normal manner and grow luxuriantly under suitable conditions as stated above. They also produce the amount of gas characteristic for these organisms.

Frequently, if one inoculates a second set of fermentation tubes from the sediments of these abnormal types in which there is little or no gas, the gas volume will be greatly increased in amount in the second set and there will be perfect accord between the gas volume and the types of bacteria found in the sediments, namely, a representative number of Gram-negative bacilli of the colon-aerogenes type.

The explanation which seems to be the most logical and which appears to be substantiated by cultural experiments is as follows: In the first place, there is evidence that colon bacilli frequently proliferate in fermentation tubes until a very decided turbidity is observed in both the closed arm and the bulb before gas production becomes apparent, or, in other words, a consider-

able vegetative development precedes visible gas formation. One sees in the sediments of such tubes perfectly staining, large, well-developed colon bacilli grown in a favorable environment, whereas in the feces one finds similar organisms which stain faintly, due doubtless to the unfavorable environment to which the latter bacteria were subjected.

Thus we may state the case as follows: In the feces from which the bacteria in these abnormal fermentation tubes were derived the acidophilic bacteria were either much more numerous than the colon bacilli, or there were present strains which could vegetate much more rapidly in artificial media than these varieties usually met with. (This statement is well substantiated by actual investigation. One has only to isolate a few cultures of acidophilic bacteria to appreciate the differences between various strains with respect to their ability to grow in even the most favorable artificial media. Some of these bacteria grow luxuriantly from the start, while others do not become acclimated until many transfers have been made.)

Almost as soon as the feces are sown into fermentation tubes, the colon bacilli commence to proliferate. At the same time the acidophile bacteria, either because of their unusual numbers, or because they are of such a nature as to grow more rapidly than those ordinarily met with (or owing to a combination of both of these factors) also begin to develop rapidly. The acidophilic bacteria produce acid in considerable amounts, and rapidly. The colon bacilli also produce acid, and the combined acidity is sufficient in a comparatively short time to prevent the further growth of the colon bacilli, and consequently to inhibit the production of gas. The number of organisms which have already grown, however, is sufficient to form a very fair proportion of the total flora of the sediment. As a result one sees the picture described above, namely, the presence of moderate numbers of Gram-negative bacilli, morphologically (and culturally) *B. coli*, associated with a minimal gas volume.

It is possible that there may be other explanations for this phenomenon, but the one presented is substantiated by cultural experiments, in so far as it is possible to imitate artificially the conditions which bring about this state of affairs. It is not practicable, however, to imitate this condition with absolute regular-

ity in artificial media, chiefly because it is not possible to so govern the relative rate of growths of the two types of bacteria that the sequence of events shall be as above outlined.

NOTE: Another bacterial combination, not mentioned above, results in an increased volume of gas in the bioses, particularly saccharose. Some strains of *B. coli* do not produce gas in saccharose, but in the presence of certain kinds of bacteria which secrete invertin, gas is produced, but more slowly than is usually the case. What has actually happened is that the colon bacillus acts upon the dextrose and laevulose which in turn are derived from the saccharose by the inverting action of the associated organism. This may actually be reproduced, for example, by combining certain acidophilic bacteria with *B. coli*. The former grow slowly and produce a certain amount of easily fermentable substance from the saccharose. As soon as this simple sugar is produced, the non-saccharose-fermenting strain of *B. coli* begins to form gas. The amount of gas generated, however, is always less than would be the case with the same organism in dextrose or lactose, or in parallel cultures using colon bacilli fermenting saccharose. This gas production, it should be stated, is not brought about by the action of colon bacilli upon products of the saccharose from sterilization nor by the accidental presence of hexoses in the saccharose as impurities. One finds in control tubes from the same lot of fermentation media (saccharose) that the colon bacillus by itself cannot produce gas, even if the experiment is prolonged a week.

SUMMARY.

Generally speaking the gas volume in fermentation tubes inoculated with infantile fecal flora depends upon the relative vegetative activity of three types of bacterial forms: the aërogenic (*B. coli*, *Bact. aërogenes*, rarely *B. paratyphi*), the acidogenic (*B. bifidus*, *B. acidophilus*, coccal forms), and those organisms which produce alkali.

The colon-aërogenes bacilli are the common gas-forming organisms of the mixed fecal flora; if they are not present or if their vitality be depressed, little or no gas will be produced in fermentation tubes inoculated with feces. The amount of gas which these aërogenic bacteria can generate is in general diminished by the unrestrained activity of acidophilic bacteria, simply because these acid-producing bacteria render the medium unfavorable for the continued proliferation of the colon group. On the other hand, certain alkali-producing bacteria which can neutralize the acid produced by either the colon group or the acidophilic

group render the medium more fitted for the development of the colon bacillus, or at least permit these latter bacilli to grow for a longer time, thus resulting in an increase in the amount of gas formed.

Not all alkali-producing bacilli, however, will grow symbiotically with the colon bacillus. Certain strains actually inhibit the vegetative reproduction of the aërogenic bacteria, particularly in dextrose and saccharose.

Lactose, generally speaking, is less readily attacked by the facultative intestinal organisms. This is more marked in the case of the so-called "wild races," many of which actually produce alkali in lactose, although they generate small amounts of acid in dextrose and saccharose (thus indicating that the lactose cannot readily form a part of their nutriment). In lactose, consequently, the restraining action of these bacteria may be less marked than is the case with dextrose or saccharose. This does not, however, always result in the production of a greater volume of gas in lactose than is the case with the other two sugars: the gas volume may be greater in dextrose and saccharose, as has been indicated above. The amount of gas produced in lactose is, on the whole, more constant than is the case with either dextrose or saccharose, and, broadly speaking, the gas volume and the character of the Gram-stained sediments in this carbohydrate are better indices of the relative viability of the *obligate* intestinal flora than are either of the other two carbohydrates.

Having established presumptive explanations, based upon experimental data for these observed irregularities in fermentative action of the mixed fecal flora, it is essential to correlate these findings with their antecedent agents in the intestinal tract.

It will be necessary, in order to present the facts logically, to review briefly the distribution of bacterial types in the alimentary canal.

Broadly speaking, upon a mixed diet the upper levels of the tract—from the lower duodenum to the ileum—are colonized by considerable numbers of bacteria of the subtiloid type—organisms which liquefy gelatin, peptonize casein, do not form gas in carbohydrates, but which may produce small quantities of acid in dextrose and saccharose. The reaction is usually alkaline in lactose. In dogs, with a diet rich in proteids, the habitat of these

organisms is extended downward in the tract to the cecum and the higher levels of the colon, although as a rule they are relatively less abundant in this region than in the small intestine.

Escherich[1] noticed that in dogs with a protein-rich diet there was a decided increase in the liquefying forms occurring in the feces, and his experiments in this direction are substantiated by the data presented above.

Coccal forms are usually most abundant in the region of the jejunum, particularly the lower half, and the ileum.

Aërogenic bacilli, chiefly *B. coli* and its variants, are most numerous from the cecum to the transverse colon, while the acidophiles and anaërobes are most frequently met with in the descending colon and rectum, where the anaërobic conditions are most marked.

A diet rich in protein, particularly meat (in dogs) and relatively poor in easily fermentable carbohydrates is therefore attended by an extension of the habitat of the liquefying bacteria, and usually by an increase of the aërogenic bacilli (*B. coli*) while the growth of acidophilic bacteria is restricted. In such instances there is usually a decided augmentation of the gas volume above that characteristic of a mixed diet: the sediments show increased numbers of subtiloid bacilli[2] and aërogenic organisms, while the acidophiles are relatively poorly represented.

A carbohydrate-rich diet, on the other hand (particularly if the carbohydrates are easily fermentable sugars rather than starches) results in a limitation of the liquefying bacteria, and a more or less marked restriction of the development of the aërogenic forms accompanied by a decided increase in the acidophiles. The gas volume is decreased and in the sediments are seen the acidophilic bacteria prominently represented, while the other types are suppressed to a greater or less extent.

While, in general, the above statements are substantiated by actual experiments, the results may be considerably modified by the length of time during which the intestinal contents sojourn in the various levels of the tract, and in interpreting results one should be fully informed upon this point. If the fecal mass

[1] Escherich: *Jahrb. f. Kinderheilk.*, lii, p. 1, 1900.

[2] Cf. Kendall: *loc cit.*

passes through the colon rapidly, and is voided promptly, the acidophilic bacteria do not have time to proliferate to such an extent as is the case under normal conditions, and the resulting gas volume may be relatively increased, even upon a diet rich in carbohydrates.

It will not be possible in this paper to go more into detail with reference to the relation of gas volume and character of the sediment to diet. Enough has been said to indicate in a general way the fundamental relations between the character of the intestinal bacteria and the phenomena which they originate in the fermentation tubes.

Remembering that the bacteria from the higher levels of the alimentary canal develop more rapidly as a rule in artificial media (fermentation tubes) than do those of the lower colon and rectum, it will be readily comprehended that those factors which limit the development of the acidophilic bacteria in the alimentary tract will tend to increase the gas volume rapidly, while the restriction of the gas volume, on the other hand, in general depends upon the activity of relatively large numbers of acidogenic forms (because these organisms develop more slowly in artificial media than do those from the higher levels).

THE METABOLISM OF MAN DURING THE WORK OF TYPEWRITING.

By THORNE M. CARPENTER and FRANCIS G. BENEDICT.

(From the Nutrition Laboratory of the Carnegie Institution of Washington, Boston, Mass.)

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In the present stage of civilization and education, the work of handwriting becomes a part of the every-day routine of a very large number of people, and in attempting to estimate the energy transformations which take place during a day of ordinary routine some idea of the amount of energy involved in writing is of importance. In all probability, the energy transformations involved in handwriting are not very large. There is considerable muscle tonus in the one arm and hand and there may be a sympathetic muscular tonus in the other, but when compared with the ordinary muscular movements in every-day life, one would not expect wide variations.

Unfortunately we have at present practically no quantitative data regarding this rather important phase of every-day life. Speck,[1] in studying the respiratory exchange with his spirometer and mouthpiece, included some experiments in which there was more or less handwriting, but these experiments were so complicated with other extraneous influences that they have but little value in indicating the nature of the metabolism as influenced by handwriting.

Wolpert[2] reports four experiments with a writer using the increment in carbon dioxide excretion over rest to compute the work done in handwriting. Unfortunately the amount of work accomplished, the previous diet, the time of day and the pulse rate are not given. In his experiments, two resting and two copying out of a book, there was a carbon dioxide excretion during rest of 33.844 and 30.790 grams per hour and during work an hourly

[1] C. Speck: *Physiologie des menschlichen Athmens*, Leipzig, 1892, p. 204.
[2] *Arch. f. Hygiene*, xxvi, p. 68, 1896.

excretion of 37.412 and 37.986 grams. Using the values obtained in previous experiments on work Wolpert calculates that each gram increase in the excretion of carbon dioxide corresponds to 300 kilogram-meters. From this he concludes that his subject during writing produced 1600 kilogram-meters per hour.

Singularly enough, this amount of work corresponded almost exactly to that accomplished by a tailor also studied by Wolpert. The body weight of the tailor (49 kilos) was, however, much less than that of the writer (64) kilos. Since one calorie is equivalent to 427 kilogram-meters, the extra heat production when writing is about 4 calories per hour.

In connection with a series of experiments recently reported by us,[1] a certain amount of information was obtained regarding the work of handwriting of a number of students during control experiments designed for comparison with metabolism experiments during severe mental strain. In these control experiments, which lasted some three hours, the students were required to duplicate during the first hour and a half the larger proportion of the actual amount of writing done on the examination paper during the examination period. During the experiments with the examinations, the larger amount of writing was done immediately after the beginning of the experiment and a small portion of it accomplished in the second one and a half hour period. Similarly, in the control experiments there was a period of one and a half hours in which there was a considerable amount of handwriting and a period immediately following in which there was a much smaller amount of handwriting, although the difference is not as sharp as could be desired for making definite deductions. A comparison of the metabolism in these two periods shows that there was a somewhat increased metabolism during the first period. Considering the question from the heat elimination alone, there were some 159 calories eliminated in the first period as against 146 in the second. In inspecting these figures, however, several factors should be taken into consideration. In the first place, it is not a comparison of a period with handwriting as against a period without handwriting, as there was some hand-

[1] On the Influence of Muscular and Mental Work upon Metabolism and the Efficiency of the Human Body as a Machine, Bulletin 208, Office of Experiment Stations, U. S. Department of Agriculture, 1909.

writing done in both periods. Secondly, the work of digestion, which plays a not unimportant rôle in resting experiments of short periods, was undoubtedly greater during the first hour and a half period than during the second. Under these circumstances it is hardly permissible to state definitely that the handwriting did produce a material increase in the metabolism, since the experiments were not designed to study this point and they are not sharply enough drawn to warrant positive inferences.

In the past thirty years there has been a marked development in the use of mechanical machines for writing and the typewriter of the present day furnishes a machine with which there can be made an extremely rapid written record. The major movements involved in writing a word on the typewriter are, however, very much greater than those involved in writing a single word with the pen, and while there is a marked increase in speed over the handwriting with the pen, there must obviously be a much greater increase in the muscular activity involved in recording material in this manner. Indeed, *a priori*, we may assume that there is a much greater increase of metabolism as a result of using a typewriter when compared with handwriting, even on the basis of the energy elimination per 100 words.

There has been an extensive introduction of machine writing and practically every business office is today supplied with machines of this nature. The operators of these machines are in large part women who are not muscularly as well developed as is the ordinary man; in fact, there is no particular evidence to indicate that the women using these machines are muscularly more developed than the average woman, and yet they are able to accomplish an enormous amount of work with no excessive fatigue. It, therefore, becomes important in estimating the energy requirements of a user of one of these machines to have some idea of the amount of energy required for writing with this appliance. The problem is of great interest and serves admirably to indicate the possibilities of the use of accurate scientific instruments for studying problems having a practical every-day value. One such problem has recently been studied by Reach[1] in Durig's

[1] Untersuchungen über die Arbeitsleistung des Menschen mit besonderer Rücksichtnahme auf ihre praktische Beziehung zum Betrieb landwirtschaftlicher Maschinen, *Land. Jahr., Zeitschrift für wissenschaftliche Landwirtschaft*, pp. 1053 to 1101. 1908.

laboratory in Vienna, namely, the problem of the application of the hand lever in rotating a milk separator. It is clear, then, that problems of this nature may be expected to be developed more in the immediate future than heretofore, and it is interesting to note that at the present moment the Department of Agriculture in Washington is constructing a respiration calorimeter on the same plan as that formerly at Wesleyan University, with which it proposes to take up problems of exactly this nature.

The observations here reported were made in connection with a series of experiments to study the normal metabolism of resting man. To diminish as much as possible the *ennui* experienced by a long sojourn inside the respiration chamber, one subject with whom two experiments had been made utilized a small portable typewriter for recording his impressions and writing letters during his stay inside the chamber. No attempt was made to estimate the amount of work done in this manner, as there were at that time no experimental data available. The suggestion was made, however, that a knowledge of such energy transformation as is required by the use of a typewriter would be of extremely practical value not only in determining the results of experiments made in which typewriting was done inside of the chamber, but also as throwing light upon the energy requirements of persons using the machine in every-day life. Accordingly opportunity was had to study two men who had experience in using the typewriter and in connection with the study of their normal resting metabolism, an experiment in which typewriting was done was appended to each test. Obviously, two experiments such as are here reported can in no wise throw any conclusive light upon the possible transformations of energy attending the use of these typewriting machines. There may be variations in the different types of machines, depending upon the weight of the carriage, distance through which it travels, height to which it is raised, strength required to strike the keys, and in tension; there may be a marked difference in the different systems of fingering in which there is a difference in the amount of muscular activity required to adjust the hand to the keyboard, and, finally, there would unquestionably be large variations in individuals in regard to nervous tension and muscular tonus during such a test. Pending, therefore, a more elaborate study

of problems of this nature, it seems desirable at this time to give the results of the two preliminary experiments.

EXPERIMENTAL PART.

Plan of experiments: The general plan of the experiments was similar to that followed in the series of experiments on the normal metabolism of resting man begun in the laboratory of Wesleyan University and continued over several years. The plan was essentially as follows:

The subject entered the chamber usually after a meal and sat quietly in an arm-chair, with a table before him, reading for an hour or so until the calorimeter was in perfect thermal equilibrium. At this time the analyses of the residual air were made and the heat measurements began. During the resting period the man sat quietly reading. During the typewriting period he used the typewriter which had previously been placed in the chamber, the total number of words written and the time occupied being accurately recorded.

Apparatus used: The experiments were made inside the respiration calorimeter at Wesleyan University, which apparatus has been described in detail in several places.[1] It is sufficient to state here that it consisted of an air-tight, fairly well lighted chamber with double metal walls, in which the subjects could sit comfortably, and the ventilation was maintained by a rotary blower which removed the vitiated air, forced it through purifiers containing sulphuric acid to absorb the water vapor and through soda lime to remove the carbonic acid, and returned it again to the chamber, the deficiency in oxygen being made up by admitting oxygen from a cylinder of the highly compressed gas. The heat given off by the subject was measured by a current of cold water passing through the chamber, due precaution being taken for the prevention of heat passing through the walls by the excel-

[1] A Respiration Calorimeter with Appliances for the Direct Determination of Oxygen, W. O. Atwater and F. G. Benedict, Publication No. 42 of the Carnegie Institution of Washington, 1906.

Experiments on the Metabolism of Matter and Energy in the Human Body, F. G. Benedict and R. D. Milner, Office of Experiment Stations, U. S. Department of Agriculture, Bulletin 175, 1907.

lent adiabatic scheme of Rosa[1] and allowing for the amount of heat required to vaporize the water.

Determinations made: In these experiments of short duration, no attempt was made to strike a complete balance of intake and outgo. Usually the subjects prior to the experiment partook of unknown amounts of food. In these experiments the ur ne was not collected, and the feces passed were not taken into consideration, although it was usually planned to avoid defecation during the experiments, either by having the subjects defecate before the experiments or else by emptying the lower bowel with a warm-water enema. The actual determinations, then, that are of value are the water vaporized by the lungs and skin, the carbon dioxide exhaled, the oxygen consumption, the heat elimination, and, where possible, the heat production. In certain experiments, also, the loss in body weight was accurately recorded. From the water vaporized, the carbon dioxide excretion and oxygen absorption, it is possible to compute approximately the total catabolism. Particularly is this so if the nitrogen excretion is exactly known; when it is not, the average amount of nitrogen excretion under similar conditions is used and found to give results well within the limits of experimental error. The direct measurements of heat indicating the true energy transformations independent of calculations of the gas exchange lend an added interest to the value of the experiments.

Experiment No. 1: Subject, C. F. S.; October 24, 1905. The subject, whose naked body weight was 60.1 kilos, was 170 cm. high. He was 35 years of age, and had formerly been an expert typewriter in the Canadian government service, having taken prizes for excessive speed in competition. Subsequently he had been a practising lawyer and had used his typewriting only incidentally, and so did not consider himself by any means in expert form. Furthermore, the machine available for his use at this time, although of a well-known make, was one with which he was not entirely familiar.

[1] Description of a New Respiration Calorimeter and Experiments on the Conservation of Energy in the Human Body, W. O. Atwater and E. B. Rosa, Office of Experiment Stations, U. S. Department of Agriculture, Bulletin 63, 1899.

He entered the respiration chamber in the forenoon at about 9 o'clock and the experiment proper began at 10.20 a.m. During the first two hours he sat quietly reading, sitting in an arm-chair and making no extraneous muscular motions. The temperature was taken in the mouth by a mercurial thermometer at the beginning of the experiment, at the end of the first period, and at the end of the experiment. The temperature remained constant at 98.2° F. throughout the whole experiment.

During the experiment the subject was asked to take the pulse from time to time, counting it for two minutes and recording it himself. At the end of the first 2-hour period, the subject went to the food aperture in the respiration chamber, took the food therefrom and ate it rather hurriedly. The food consisted of 90 grams of graham crackers and 450 cc. of milk. Shortly after eating lunch, he began working with the typewriter and continued more or less steadily during the remaining two hours, stopping occasionally for a moment or two.

The pulse record during the experiment was as follows:

10.28 a.m.	74
11.00	69
11.28	66
12.00	68
12.25 p.m.	67
2.20	77

This experiment was the first experience of the subject inside the respiration chamber and the novelty of the situation produced in him, as it does in most subjects, a slight stimulation. During the two hours he wrote 3306 words, writing from memory, legal documents, poems and miscellaneous material. In commenting upon the experiment, he reported that he was very comfortable and there were no sensations out of the ordinary to record. While reading he felt a little cool, and after he had been writing for an hour felt very warm and perspired freely. The machine was a little strange to him at the start and he feared that he would not be able to write enough to make the test of any definite value. With regard to the temperature changes inside the chamber, it may be well to add that the control of the temperature inside the chamber is such that the air seldom varies 0.05° C. and usually is inside this limit.

Carbon dioxide elimination: During the experiment the carbon dioxide elimination was measured in 2-hour periods, the one of rest and one during the work. To the total amount of carbon dioxide absorbed in the soda lime absorbers is added or subtracted the carbon dioxide withdrawn or remaining in the chamber over the preceding period, thus making due allowance for the variations in the composition of the air inside of the chamber. The carbon dioxide data are given in Table 1, which shows that there was a marked increase in the carbon dioxide production accompanying the work.

TABLE 1

Carbon dioxide exhaled by C. F. S. in experiment during rest and typewriting.

DATE	PERIOD	RESIDUAL AMOUNT IN CHAMBER	DIFFERENCE	AMOUNT IN ABSORBERS	TOTAL EXHALED	VOLUME EXHALED BY SUBJECT
1905		*grams.*	*grams.*	*grams.*	*grams.*	*liters*
Oct 24...	Preliminary.....	38.6				
" 24...	10.18 a.m.–12.18 p.m. Rest	39.9	+1.3	48.6	49.9	25.4
" 24...	12.18 p.m.–2.18 p.m. Typewriting.	49.8	+9.9	68.4	78.3	39.9

Water elimination: The total water vaporized inside the chamber is measured by the apparatus but is of especial value as indicating the amount of heat required to vaporize this water, thus permitting a very close measurement of the actual amount of heat eliminated during the experiment. The data for the water are given in Table 2.

TABLE 2

Water vaporized by C. F. S. in experiment during rest and typewriting.

DATE	PERIOD	RESIDUAL AMOUNT IN CHAMBER	DIFFERENCE	AMOUNT IN ABSORBERS	TOTAL WATER VAPORIZED
		grams.	*grams.*	*grams.*	*grams.*
Oct. 24..	Preliminary.....	53.9			
" 24..	Rest.....	49.9	− 4.0	73.3	69.3
" 24..	Typewriting.....	64.8	+14.9	75.3	90.2

To the total amount of water absorbed out of the air current as it is forced through the concentrated sulphuric acid in the absorbing vessel, is added or subtracted the differences in the amount of the water vapor remaining in the chamber.

There is a noticeable increase in the amount of water residual in the chamber at the end of the second period. There is likewise a marked increase in the total amount of water given off. This is in conformity with the subject's idea that he perspired freely during the second period, although in a number of other experiments published from this laboratory, it has not been found invariably the case that an increased water elimination accompanies a feeling of increased perspiration.

Oxygen absorption: Of great value for indicating the complete metabolism of material in the body is the true measurement of oxygen absorption. This was possible by means of the apparatus used in these experiments which permitted the direct determination of oxygen. In determining the oxygen, not only is the loss in weight of the steel cylinder of the highly compressed gas recorded as indicating the amount of oxygen admitted to the chamber but also there are differences in the residual amount of oxygen in the air that must be taken into consideration. These may be considerable owing to the variations in barometric pressure. Thus it is shown in the data in Table 3 that there is a decrease of some 31 liters of oxygen in the chamber at the end of the second period as compared with the beginning. The total oxygen consumption was considerably greater during the working period than during the rest.

TABLE 3

Oxygen absorbed by C. F. S. in experiment during rest and typewriting.

DATE	PERIOD	RESIDUAL AMOUNT IN CHAMBER	DIFFERENCE		AMOUNT ADMITTED TO CHAMBER	TOTAL AMOUNT CONSUMED BY SUBJECT	VOLUME OF CARBON DIOXIDE EXHALED	VOLUME OF OXYGEN CONSUMED	RESPIRATORY QUOTIENT
		liters.	*liters.*	*grams.*	*grams.*	*grams.*	*liters.*	*liters.*	
Oct. 24	Preliminary	909.5							
" 24	Rest	907.2	−2.30	− 3.3	40.9	44.2	25.4	30.9	0.82
" 24	Typewriting	876.3	−30.9	−44.0	26.0	70.0	39.9	49.0	0.81

The relation between the carbon dioxide elimination and the oxygen consumption (the so-called respiratory quotient) is of great value in indicating the nature of the material burned. During these two periods, singularly enough, the respiratory quotient was nearly the same, 0.82, as compared with 0.81. In the first period, the subject had had no food since breakfast. Unfortunately no accurate record of this meal was kept. During the second period, he consumed the graham crackers and milk as recorded above. Apparently, then, even with the increased carbohydrate ingestion, the respiratory quotient did not rise, although the amount of carbohydrate eaten was not very large.

Heat elimination: Of great value in experiments of this kind is the direct determination of heat, which is made possible by the calorimetric features of the apparatus. The heat may be brought away from the chamber in a number of ways; first, by the water current passing through the heat absorbers inside of the chamber, second, through the air current in the form of vaporized water, and finally, there is a sensible heat of excreta which, however, in experiments with the calorimeter, is usually allowed to escape into the room until the excreta have attained the temperature of the chamber. The heat determinations for this experiment are recorded in Table 4, in which the first column shows the heat measured by the water current, the second, the heat required to vaporize the water, the third, the heat stored or lost from the chamber by virtue of changes in temperature, this involving obviously a knowledge of the hydrothermal equivalent of the calorimeter (a value assumed as 60 calories), then a correction

TABLE 4

Heat produced by C. F. S. in experiment during rest and typewriting.

DATE	PERIOD	HEAT MEASURED	VAPORIZATION OF WATER	CORRECTION FOR CHANGE OF CALORIMETER	CORRECTION FOR FOOD AND DISHES	CORRECTED HEAT MEASURED	BODY CORRECTION*	HEAT PRODUCED
		calories.	*calories.*	*calories.*	*calories.*	*calories.*	*calories.*	*calories.*
Oct. 24.	Rest.....	127.5	40.7	+6.0		174.2	−1.1	173.1
" 24.	Typewriting.....	172.3	52.8	−2.4	3.3	226.0	+6.3	232.3

* Correction for body weight and body temperature.

for the temperature of the food and dishes, and finally a correction for the changes in body weight. The final heat production indicates 173 calories for the first or resting period and 232 for the working period.

A comparison of all these factors of metabolism is shown in Table 5, in which it is seen that there is an appreciable increase in catabolism attendant upon working with a typewriter. A close inspection of the figures shows, however, that there is a lack of agreement between the increment of the heat production when compared with the increment of the carbon dioxide elimination and oxygen consumption. If the carbon dioxide elimination or oxygen consumption is to be taken as an index of heat production, there is an apparent discrepancy and pending a complete and elaborate discussion of this subject in a subsequent publication, it may be stated that a large number of other experiments conducted with the respiration calorimeter at Wesleyan University and, indeed, with the new calorimeters at the Nutrition Laboratory in Boston, indicate that in short periods of six hours or less, there are at times inexplicable differences between direct and indirect calorimetry. The lack of uniformity found in this and the following experiment here reported is in full conformity with the experience in other similar tests and, indeed, in tests specially devised for studying this particular problem.

TABLE 5

Comparison of factors of metabolism in experiment with C. F. S. during rest and typewriting.

DATE	PERIOD	CARBON DIOXIDE EXHALED	WATER VAPORIZED	OXYGEN ABSORBED	HEAT PRODUCED
		grams.	*grams.*	*grams.*	*calories.*
Oct. 24	Rest	49.9	69.3	44.2	173.1
" 24	Typewriting	78.3	90.2	70.0	232.3

Experiment No. 2: Subject, W. C. A.; age, 21 years; weight, 69.2 kilos; and height, 183 cm. The subject was a student at Wesleyan University who had partially earned his way through college by doing typewriting. He had had considerable experi-

ence with a machine of a standard make which he owned himself and this machine was used in the experiment. The subject entered the chamber at 3.30 p.m., October 24, a few hours after the subject of the preceding experiment had left it. The experiment proper began at 5.08 p.m.

For the first three hours he sat for the most part reading quietly although a lunch consisting of 150 grams of graham crackers and 461 grams of milk was eaten between 6.08 and 6.28 p.m. Preceding the lunch he left the chair in which he had been sitting and went to the food aperture, removing the milk and crackers. At 7.20 p.m., he rose from the chair and passed urine. At 8.08 p.m., the work experiment began and for the three hours he spent most of the time using the typewriter with but a change now and then of stopping to take the pulse. At 10.20 p.m., he went to the food aperture and removed therefrom a bottle of water which he drank; the experiment terminated at 11.08 p.m. During the first hour he wrote 1525 words, during the second 2235, and during the third 1996 words.

The pulse as recorded by the subject is as follows:

5.30 p.m.	54
7.50	68
8.00	67
9.20	72
10.20	65
11.15	64

This was also this subject's first experience inside the respiration chamber and he reported that after entering the chamber at 3.30 p.m. and while the window was being sealed in, he felt uncomfortably warm but that thereafter the air seemed clear and invigorating. He had no feeling of sleepiness or weariness during the entire experiment; he enjoyed the working period and felt more ready for work at the end than he did at the beginning. He felt as hungry as he would have out of doors and ate 16 graham crackers and drank 450 cc. of milk. He also drank 153 cc. of water about half an hour before coming out of the chamber.

Carbon dioxide elimination: The carbon dioxide elimination along with the total metabolism was determined in two 3-hour periods, one covering the rest period and one the working period. The results are recorded in Table 6.

TABLE 6

Carbon dioxide exhaled by W. C. A. in experiment during rest and typewriting.

DATE	PERIOD	RESIDUAL AMOUNT IN CHAMBER	DIFFERENCE	AMOUNT IN ABSORBERS	TOTAL EXHALED	VOLUME EXHALED BY SUBJECT
1905		*grams.*	*grams.*	*grams.*	*grams.*	*liters.*
Oct. 24..	Preliminary.....	32.4				
" 24..	5.08 p.m.–8.08 p.m. Rest.	47.5	+15.1	75.6	90.7	46.2
	8.08 p.m.–11.08 p.m. Typewriting.	48.0	+ .5	119.8	120.3	61.2

There was a marked change in the amount of carbon dioxide residual in the chamber at the end of the first period and the total carbon dioxide given off increased some 30 grams as a result of the work.

Water elimination: The total amount of water vaporized is recorded in Table 7. Here the residual amounts changed as during the experiment with C. F. S., there being an increase of some 10 grams during the working period. In this instance, however, there was hardly an appreciable increase in the total water given off as during the working period there was about 5 grams more water vaporized than during the rest period.

TABLE 7

Water vaporized by W. C. A. in experiment during rest and typewriting.

DATE	PERIOD	RESIDUAL AMOUNT IN CHAMBER	DIFFERENCE	AMOUNT IN ABSORBERS	TOTAL WATER VAPORIZED
		grams.	*grams.*	*grams.*	*grams.*
Oct. 24..	Preliminary.....	52.4			
" 24..	Rest.....	48.7	−3.7	112.1	108.4
" 24..	Typewriting.....	58.3	+9.6	104.2	113.8

Oxygen Absorption: The oxygen consumption is recorded in Table 8.

TABLE 8.

Oxygen absorbed by W. C. A. in experiment during rest and typewriting.

DATE	PERIOD	RESIDUAL AMOUNT IN CHAMBER	DIFFERENCE		AMOUNT ADMITTED TO CHAMBER	TOTAL AMOUNT CONSUMED BY SUBJECT	VOLUME OF CARBON DIOXIDE EXHALED	VOLUME OF OXYGEN CONSUMED	RESPIRATORY QUOTIENT
		liters	*liters*	*grams*	*grams*	*grams*	*grams*	*liters*	
Oct. 24	Preliminary.....	904.8							
" 24	Rest.....	903.9	− 0.9	− 1.3	79.8	81.1	46.2	56.7	0.81
" 24	Typewriting....	888.9	−15.0	−21.5	89.8	111.3	61.2	77.9	0.79

There was a marked decrease in the total oxygen content of the residual air during the second period. The effect of the work was to increase the total oxygen consumption some 30 grams. The respiratory quotient remained remarkably constant during the two periods, indicating that the nature of the material burned was much the same in both periods.

Heat elimination: The heat elimination as measured in the two 3-hour periods is recorded in Table 9.

TABLE 9.

Heat produced by W. C. A. in experiment during rest and typewriting.

DATE	PERIOD	HEAT MEASURED	VAPORIZATION OF WATER	CORRECTION FOR CHANGE OF CALORIMETER	CORRECTION FOR CHANGE OF ABSORBERS	CORRECTION FOR FOOD AND DISHES	CORRECTED HEAT MEASURED	BODY CORRECTION*	HEAT PRODUCED
		calories	*calories*	*calories*	*calories*	*calories*	*calories*	*calories*	*calories*
Oct. 24	Rest.....	251.4	63.5	−2.4		+9.1	321.6	+6.5	328.1
" 24	Typewriting	286.0	66.7	+3.0	+0.9	+6.1	362.7	+2.6	365.3

* Correction for body weight and body temperature.

In this experiment there was a slight correction for the variation in the average temperature of the water inside of the heat

absorber system. The total heat production was increased some 37 calories during the typewriting period.

The same discrepancy which appeared between the increments of the carbon dioxide elimination and oxygen consumption, and the increment of heat production in the experiment with C. F. S., indicating an abnormality between the direct and indirect calorimetry, appeared also in this experiment.

A comparison of all of these factors of metabolism is shown in Table 10.

TABLE 10.

Comparison of factors of metabolism in experiment with W. C. A. during rest and typewriting.

DATE	PERIOD	CARBON DIOXIDE EXHALED	WATER VAPORIZED	OXYGEN ABSORBED	HEAT PRODUCED
		grams.	*grams.*	*grams.*	*calories.*
Oct. 24..	Rest.....	90.7	108.4	81.0	328.1
" 24..	Typewriting.....	120.3	113.8	111.3	365.3

DISCUSSION OF RESULTS.

While these experiments were designed to study particularly the influence of typewriting, other pressing scientific problems made it impossible to carry the research farther and hence but two experiments were made. Owing to the marked difference in the temperaments of the individuals, the degree of previous skill, and of practice at the time, a comparison of experiments of this nature is, at best, somewhat speculative. From a study of these data there may be derived, however, at least a general impression of the work involved in typewriting. Accordingly, the experiments have been computed on the basis of quantities per hour and are arranged in Table 11 for comparison.

With C. F. S., the result of the work on the typewriter was to increase the carbon dioxide elimination 14.1 grams; the water vapor was materially increased by some 10 grams and the oxygen consumption by about 13 grams. The heat production was increased by 30 calories per hour. As has already been pointed out this increment of heat is not proportional to the increment

TABLE 11

Comparison of results of catabolism during rest and work in two experiments with typewriting.

[Quantities per hour.]

SUBJECT	PERIOD	CARBON DIOXIDE EXHALED	WATER VAPORIZED	OXYGEN ABSORBED	HEAT PRODUCED
		grams.	*grams.*	*grams.*	*calories.*
C. F. S.	Rest	25.0	34.7	22.1	86.5
" " "	Typewriting	39.1	45.1	35.0	116.2
W. C. A.	Rest	30.2	36.1	27.0	109.4
" " "	Typewriting	40.1	37.9	37.1	121.8

of the carbon dioxide elimination and oxygen consumption, thus indicating a discrepancy between direct and indirect calorimetry.

With W. C. A., the work of typewriting increased the carbon dioxide production about 10 grams; there is no appreciable increase in the water vapor and in the oxygen consumption there was an increase of about 10 grams, while most remarkably, there was an increase of but about 12 calories in the heat production. The apparent discrepancy between these results and those with C. F. S., in part disappears, however, when the conditions under which the experiments were made are more carefully considered. Thus, with C. F. S., the resting experiment began several hours after the morning meal and hence the increased metabolism which would naturally follow the digestion of food had probably in large part ceased. During the resting period, there was no food taken. On the other hand, with W. C. A., the experiment began some three hours after the hearty mid-day meal in which there was a considerable influence of the ingestion of food and furthermore, during the same resting period, the subject partook of a liberal amount of food. Furthermore, the food was all eaten nearly two hours before the end of the resting period. Thus, probably the maximum effect of the heat resulting from the digestive processes would be there introduced. Furthermore, during this resting period, the subject, W. C. A., went to the food aperture once and stood up to pass urine and both movements would contribute perceptibly to the heat production. The

effect then of these body movements is to raise the resting metabolism of W. C. A. above the normal. The influence of the ingestion of food is to increase the heat production during the resting period of W. C. A. and the working period of C. F. S.

Of the two experiments, we are inclined to believe that the one with C. F. S. indicates more nearly the proper conditions for studying the influence of typewriting on the metabolism although the complication of the ingestion of food during the working period undoubtedly results in a heat production somewhat too high. The heat production from the two men during work periods per hour is substantially the same. This is, however, not to be expected since W. C. A. was a somewhat larger man. On the contrary those who had anything to do with the experiments noted that C. F. S. was under a much higher nervous tension and accomplished his work with much more extraneous body movements than did W. C. A. This impression is fully substantiated by an inspection of the records for the pulse.

With regard to the amount of work done, W. C. A. in the last two hours accomplished more than did C. F. S. on the average of the two hours during his experiments. The first hour with W. C. A. was somewhat less than the average for C. F. S. per hour.

Under these conditions, therefore, it seems reasonable to assume that the work of writing some 1500 to 1600 words per hour on the typewriter results in an increase over the resting metabolism of some 10 to 14 grams of carbon dioxide, 10–13 grams of oxygen and 20–30 calories of heat per hour. Of these factors of metabolism, it is highly probable that the truest factor is presented by the total energy exchange as directly measured, and hence taking into consideration all the data furnished by these two experiments, we can tentatively say that the writing of 1600 words per hour on the typewriter results in a heat transformation over and above the resting metabolism of not far from 25 calories. At present too little is known regarding the energy transformation of various every-day activities to make any striking comparison, but from the studies of the gaseous exchange made by Zuntz[1] and his associates, it has been computed that there is an hourly energy expenditure of about 160 calories over and above

[1] Cited by Lusk: *Science of Nutrition*, p. 172, 1906.

the resting maintenance requirement by a man of 70 kilos walking along a level road at a rate of 2.7 miles per hour. It is seen, therefore, that the work of typewriting calls for very much less transformation of energy than does that of ordinary walking.

THE SPONTANEOUS OXIDATION OF CYSTIN AND THE ACTION OF IRON AND CYANIDES UPON IT.

By A. P. MATHEWS and SYDNEY WALKER.

(*From the Laboratory of Biochemistry and Pharmacology, University of Chicago.*)

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Cystin is almost insoluble in water but is soluble both in acids and alkalies. Its oxidation could, therefore, be studied in such solutions. The cystin used in the experiments was prepared from horn by the modification of Mörner's method already described;[1] it had been several times recrystallized and had the typical hexagonal crystalline form. Two grams of the dried cystin were weighed into each flask and 50 cc. of the acid or alkali of known strength added; the stoppers were replaced, and the flasks shaken at room temperature, the oxidation being measured as usual by the negative pressure developed in the flask and recorded by the mercury manometer. The temperature and barometer were read at the same time as the manometer. The cystin does not oxidize spontaneously in acid, but only in alkaline solution.

The first experiments were made with an impure cystin, recrystallized once, which showed an astonishingly rapid oxidation.

EXPERIMENT I. *Oxidation of Raw Cystin.*

Two grams of raw cystin dissolved in 50 cc. of N sodium hydrate and shaken at room temperature, 20° C. The absorption of oxygen as measured by the negative pressure in the flask was as follows:

TIME SHAKEN. *Minutes.*	NEGATIVE PRESSURE. *Mm. Hg.*
10	7
25	19
72	58
138	95
172	104
208	113
296	128

[1] Mathews and Walker: This *Journal*, vi, p. 21, 1909.

No other preparation has shown so rapid an absorption as this and we are at a loss to account for it. The cystin still contained a small amount of tyrosin and other impurities, but the addition of tyrosin to pure cystin does not accelerate the oxidation. It is possible that this cystin contained some iso-cystin, which oxidizes more rapidly. We have, however, been able to accelerate the oxidation of pure cystin by the addition of iron and cyanide as will be shown later.

A. The influence of acid and alkali on the velocity of oxidation.

From Tables 1 and 2 it is clear that the pure cystin oxidizes spontaneously in the air when in alkaline solution. When perfectly pure it goes slower than when impure. In the alkali it splits off some sulphide and ammonia. The oxidation is very slow when contrasted with the oxidation of cystein in neutral solution. The absorption in $\frac{N}{2}$ sodium hydrate is hardly noticeable.

The maximum absorption is in a 2N or 3N solution and the rate diminishes again in 5N solution. This is shown in Fig. 2. The reason why the oxidation diminishes in strong alkali is possibly that offered for the similar phenomenon in the oxidation of sugar,[1] namely the lower solubility of the oxygen in strong alkali; it may, however, be due to the union of the O_2 with the hydroxyl or oxygen ions reducing the amount of active oxygen. This possibility is being investigated. The reason why the oxidation goes on in alkaline and not in acid solution is probably that the cation is stable, the anion unstable.

B. The Action of Cyanides and Iron on the Oxidation.

We expected that the addition of cyanide would retard or stop the oxidation. Instead of that the first experiments made with a preparation not entirely pure, showed an astonishing acceleration.

This experiment is plotted in Fig. 1. It shows that at first the cyanide checks the oxidation but that after about one hour

[1] Mathews: This *Journal*, vi, p. 3, 1909.

TABLE 1

Oxidation of cystin at varying concentration of alkali. Figures represent negative pressure in mm. of Hg, 2 grams of cystin and 50 cc. solution in each flask.

TIME, MINUTES.	FLASK 1. N H_2SO_4.	FLASK 2. N NaOH	FLASK 3. 2N NaOH	FLASK 4. 3N NaOH	FLASK 5. 5N NaOH	TEMPERATURE.	BAROMETER.
10	0	3	1	6	6	21.2	733
33	0	3	8	10	8	21.2	733
54	0	8	14	16	11	21.0	733
79	0	11	17	17	12	21.0	733
93	0	12	20	21	13	21.0	733
Next morning began shaking again.							
5	0	22	29	29	22	22.3	739
125	0	36.5	49	50	38	22.0	739
	Clear	Clear	Light Yellow	Yellow	Yellow		
	No odor	NH_3 odor	NH_3 odor	NH_3 odor	NH_3 odor		

TABLE 2.

Oxidation of pure cystin in alkali.

TIME.	FLASK 1 N KOH CYSTIN 2 GMS.	FLASK 2 $\frac{N}{2}$ KOH CYSTIN 2 GMS.	FLASK 3 $\frac{N}{10}$ KOH CYSTIN 2 GMS.	FLASK 4 $\frac{N}{2}$ KOH NO CYSTIN	TEMPERTURE.	BAROMETER.
10.50	+ 1*	+1	0	+1	18.5	740.5
11.22	− 3	0	+0.5	+2	18.5	741
11.42	− 5	+1	+3	+5	19.5	741.2
12.12	− 6	+0.5	+5	+5	19.8	741.2
12.32	−10	−2	+5	+7	20.0	741.5
12.58	−15	−6	+5	+6	20.0	742.0
Stopped shaking until 1.12						
1.12	−16	− 6	+3	+5	19.6	742.5
1.42	−22	−11	+3	+4	19.5	742.7
2.30	−28	−14	+4	+5.5	20.0	743.4
3.20	−37	−17	+4.5	+6	19.9	743.7
Stopped. Not shaken until 8.35 next morning.						
8.55	−63	−38	−8	−8	19.0	751.0
9.25	−62.5	−34	−4	−3	20.0	751.0

* +Signs mean that there is a positive pressure in the flasks.

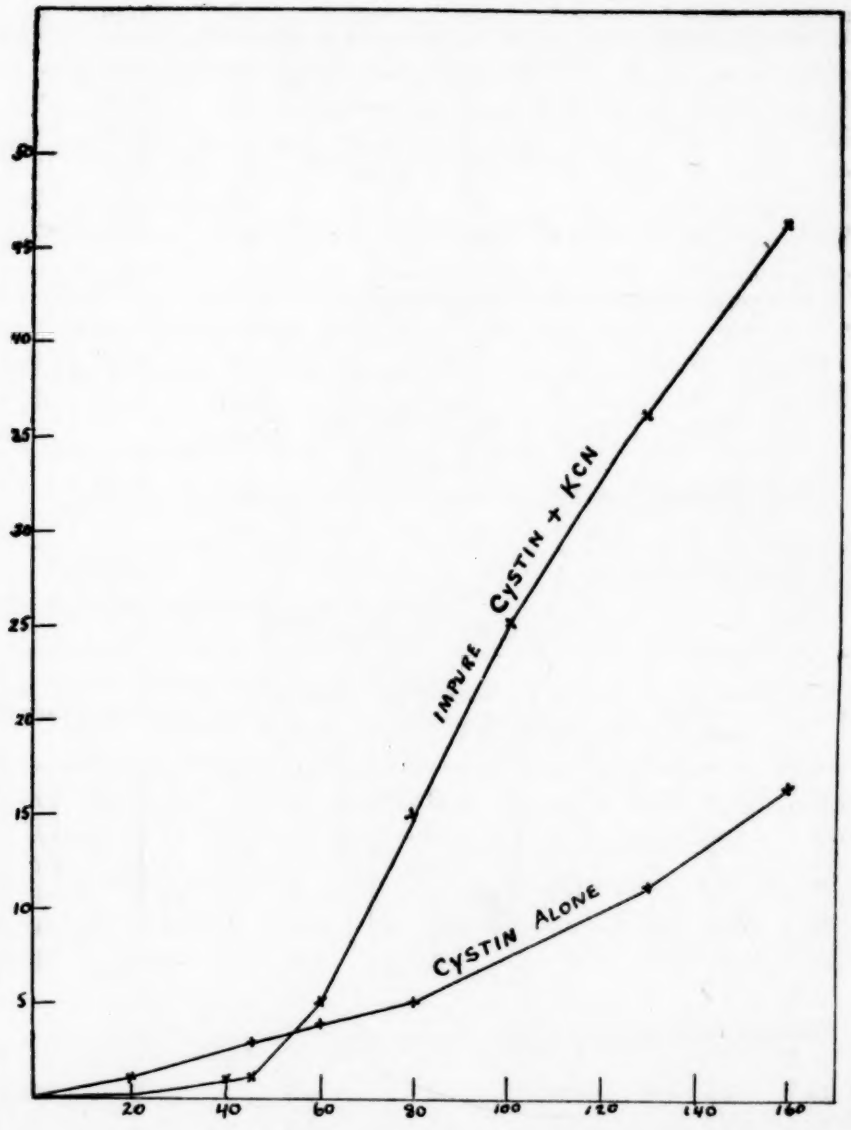

Fig. 1. Acceleration of spontaneous oxidation of impure cystin by potassium cyanide. Abscissa = time in minutes; ordinate = absorption of oxygen in mm. Hg negative pressure, N NaOH.

TABLE 3.

The accelerating action of the cyanides on impure cystin.

TIME.	FLASK 1. 2 GRAMS CYSTIN: N NaOH.	FLASK 2. 2 GRAMS CYSTIN: N NaOH + 0.1 GRAM KCN.	TEMPERATURE.	BAROMETER.
12.00	1	1	19.0	750
12.10	3	3	18.8	749.8
12.22	4	3.5	18.5	749.6
12.48	5	3.5	19.5	749.3
1.15	8	3	18.8	749.0
1.51	9	13	19.5	749.0
2.10	10	22	19.9	748.8
2.35	12	27	20.0	748.5
3.00	13	33	20.2	748.5
3.40	18	42	20.2	747.4
4.00	22	46	20.3	747.3
4.20	24	51	20.1	747.0
Stopped.	Next morning.			
8.47	43	69	18	745.5
9.26	41	72	19.1	745.5
10.15	45.5	78.5	19.4	745.8
11.42	50	85	20.9	745.5

TABLE 4.

Experimental conditions the same as Table 3.

TIME.	FLASK 1. CYSTIN ALONE.	FLASK 2. CYSTIN + 0.1 GRAM KCN.	TEMPERATURE.	BAROMETER.
1.15	0	0	19.7	745.5
1.25	1	1	20.	745.5
1.45	2	0	20.3	745.5
2.00	3	1	20.5	745.5
2.15	4	5	20.5	745.8
2.35	5	15	21.0	745.8
2.56	8	25	20.9	745.8
3.25	11	36	21	745.9
3.55	16.5	46.5	21	746.0
4.20	22	54	21	746.5
Not shaken during night. 8.43 Began shaking				
9.36	44	82	19.5	747.6

the acceleration becomes evident. We are at a loss to explain this. A second experiment gave the same result.

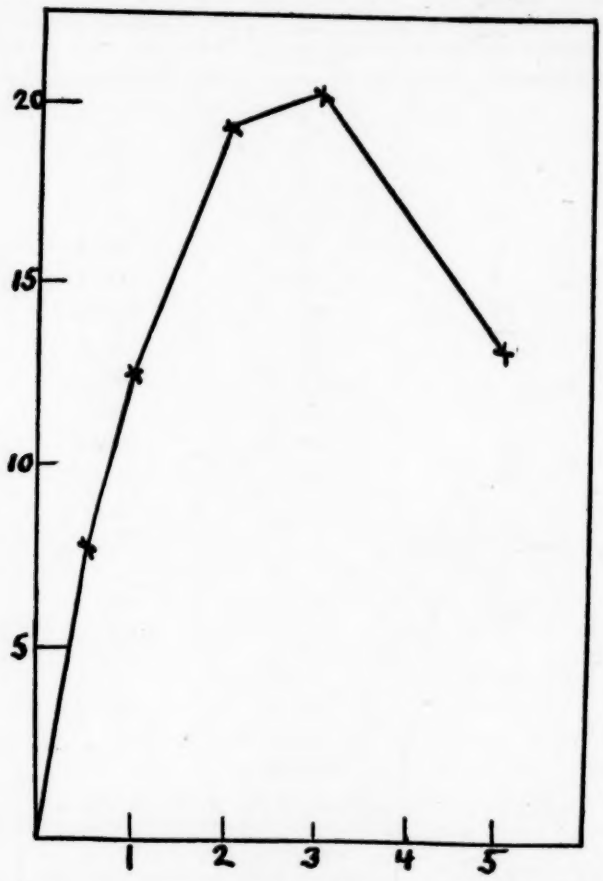

Fig. 2. Influence of concentration of alkali on spontaneous oxidation of cystin. Ordinate = oxygen absorbed in mm. Hg in 90 minutes; Abscissa = concentration of NaOH; 1–5 N.

Contrary to the foregoing the cyanide has no effect on the oxidation of pure cystin. Cystin repeatedly purified by crystallization was not accelerated in its oxidation by the addition of cyanide alone. This is shown in Table 5.

The experiment shown in Table 5 proving that cyanide had no action on perfectly pure cystin led us to look for the impurities which had caused the acceleration in the previous experiments. Our first experiments were made with the addition of ferric chloride, since the presence of a small trace of iron was not unlikely, and it had been observed that the addition of cyanide

TABLE 5.

Action of cyanide on the oxidation of pure cystin.

TIME MINUTES.	FLASK 1. 2 GMS. CYSTIN 0.05 KCN.	FLASK 2. 2 GMS. CYSTIN 0.1 GM. KCN.	FLASK 3. 2 GMS. CYSTIN 0.2 GM. KCN.	FLASK 4. 2 GMS. CYSTIN 0.5 GM. KCN.	FLASK 5. NO CYSTIN 1 GM. KCN.	TEMPERATURE.	BAROMETER.
53	0	0	0	0	0	21.3	738.6
120	0	2	3	3	0	21.0	738.0
214	2	5	3	2	0	23.0	736.0
252	4	7	6	5	0	21.6	736
Not shaken during night. Next morning 8 a.m.							
	18	20	18	15	0	21.9	736.0

in small quantities hastened the catalysis of hydrogen peroxide by iron.[1] We found that if the iron was added after the cystin was dissolved in the alkali it made no difference to the oxidation, either in the presence or absence of cyanide. This is shown in Table 6.

In this experiment the ferric chloride was added after the solution was made alkaline. Thinking that this might make a difference owing to the precipitation of the iron as the hydroxide, we tried the experiment of mixing the cystin, potassium cyanide and ferric chloride in water first and then after fifteen minutes

TABLE 6.

Each flask contained 50 cc. N sodium hydrate and 2 grams cystin.

TIME MINUTES.	FLASK 1. CONTROL. CYSTIN ALONE.	FLASK 2. 1 DROP OF 10 PER CENT $FeCl_3$.	FLASK 3. 1 DROP OF 10 PER CENT $FeCl_3$ + 0.1 GM. KNC.	TEMPERATURE.	BAROMETER.
45	4	6	4	20.8	747.7
95	7.5	8.5	6	20.8	747.7
125	9	10	10	20.8	747.6
170	13	15	14	20.6	747.7
Next morning at 8 a.m.					
	20	22	23	20.0	747.0

[1] Kastle and Loevenhart: *Amer. Chem. Journ.*, xxix, p. 419, 1903.

TABLE 7.

The action af cyanide and iron added to the cystin before the alkali. 2 grams of cystin and 50 cc. of $\frac{N}{2}$ NaOH in each flask.

TIME MINUTES.	FLASK 1. CONTROL. CYSTIN ALONE.	FLASK 2. 0.01 GM. NaCN (CONTAINS IRON.)	FLASK 3. 0.1 GM. NaCN (CONTAINS IRON.)	FLASK 4. 0.3 GM. NaCN (CONTAINS IRON.)	FLASK 5. 0.1 GM. KCN + 0.05 CC. $FeCl_3$.	TEMPERATURE.	BAROMETER.
20	0	0	0	0	0	22.8	752
80	2	3	5	2	11	22.8	752
Stopped shaking for 20 minutes.							
134	4	8	10	7	17	22.6	752
149	5	10	11	8	18	22.0	751
203	7	14	18	16	25	21.5	751
293	9	16	20	21	28	20.8	751
353	10	17	23	25	30	20.5	751
Next morning . 15 hours not shaken							
	18	25	28	33	38	22.9	748

TABLE 8.

Each flask contains 2 grams cystin and 50 cc. NaOH. Conditions of the experiment the same as in Experiment 7.

TIME, MINUTES.	FLASK 1. CONTROL CYSTIN ALONE.	FLASK 2. 0.2 GM. KCN.	FLASK 3. 0.2 GM. KCN + 1 DROP 10 PER CENT $FeCl_3$.	FLASK 4. 1 DROP 10 PER CENT $FeCl_3$.	FLASK 5. 0.2 GM. K_4FeCy_6.	TEMPERATURE.	BAROMETER.
38	0	0	6	0	1	22.5	728.2
78	2	0	9	1	4	22.1	728.2
143	2	0	11	2	4	22.5	728.2
236	4	2.5	15	3	5	23.0	728.
289	7.8	8	22.5	10	9	23.0	728.
319	9	10	24	12	10	23.0	728.
Fifteen hours not shaken.							
	20	22	37	20	19	25.2	728.

adding the sodium hydrate. We also tried the action of a sample of sodium cyanide that already contained iron as an impurity. The results are given in Table 7.

The accelerating action of the cyanide and iron together is plainly to be seen in Table 7. The same result is shown in Table 8 and Fig. 3.

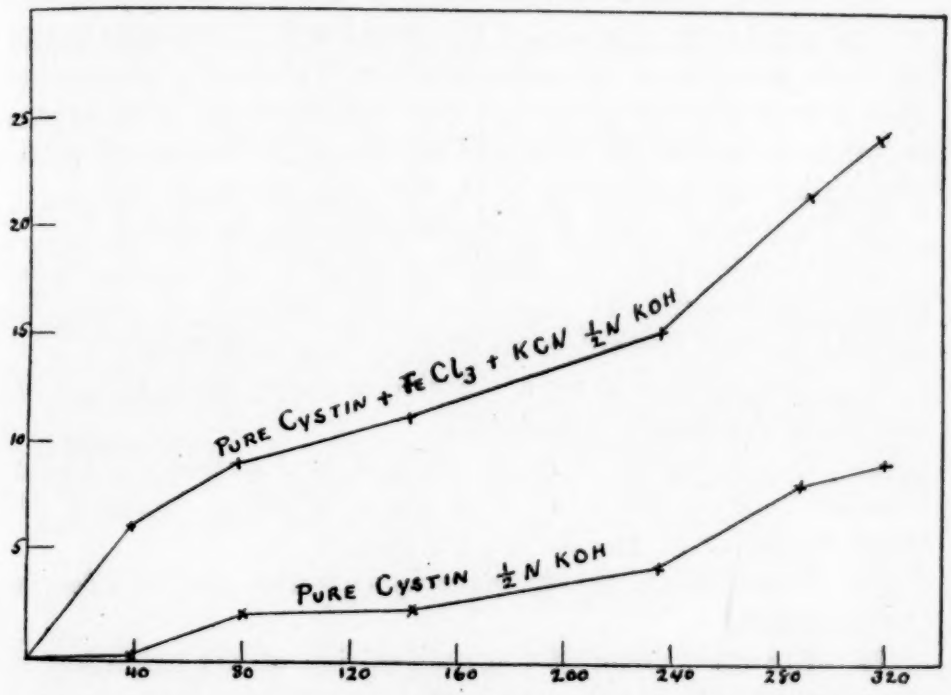

Fig. 3. Acceleration of oxidation of pure cystin by iron and cyanide. Abscissa = time minutes; ordinate = oxygen absorbed in mm. Hg.

From Table 8 it is seen that the addition of potassium ferrocyanide, of potassium cyanide alone, or iron alone has no effect in increasing the speed of spontaneous exidation of the cystin, but that the addition of iron and cyanide together increases the speed of oxidation 100 per cent or more.

We are at present unable to explain the mechanism of this acceleration, but it seems not improbable that the acceleration is due to some sort of a compound of the iron and cyanide acting as an oxygen carrier. To test this we added the ferrocyanide, but the result was negative. Apparently, at any rate, the ferrocyanide formed is not the active agent as had been sug-

gested for the acceleration of the catalysis of peroxide by Kastle and Loevenhart.[4]

SUMMARY.

(1) Pure cystin, the disulphide of α-amino-β-thio-propionic acid, oxidizes itself spontaneously in alkaline solution, but not in acid. The products of the oxidation have not been determined.

(2) The optimum concentration of alkali is between 2N and 3N sodium hydrate, 50 cc. of the solution to 2 grams of cystin. Stronger and weaker solutions of alkali oxidize at a slower rate. This is probably due to the fact that the solubility of the oxygen is less in strong alkali, or to a reduction of the amount of active oxygen by the combining of O_2 with the hydroxyl ions.

(3) The cystin anion is unstable; the cation is stable.

(4) The addition of potassium cyanide to an impure cystin greatly accelerated its oxidation. The addition of potassium cyanide to pure cystin has no effect on the oxidation.

(5) The addition of ferric chloride to the cystin solution does not affect the speed of oxidation.

(6) The addition of ferric chloride and potassium cyanide together, before the solution is made alkaline, increases the speed of oxidation 100 to 300 per cent.

(7) The addition of potassium ferrocyanide had no effect on the oxidation.

(8) The mechanism of the acceleration of the oxidation by iron and cyanide is not explained.

[4] Kastle and Loevenhart: *loc. cit.*

THE ACTION OF METALS AND STRONG SALT SOLUTIONS ON THE SPONTANEOUS OXIDATION OF CYSTEIN.

By A. P. MATHEWS and SYDNEY WALKER.

(*From the Laboratory of Biochemistry and Pharmacology, University of Chicago.*)

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The preceding papers[1] have shown that in its optimum of alkalinity, susceptibility to changes in alkalinity, to cyanides and nitriles, the spontaneous oxidation of cystein bears many resemblances to the oxidations in living matter. The present paper shows that the resemblance extends, also, to the action of iron salts and that by its relation to other metals, strong salt solutions, arsenic and mercury, this oxidation may furnish us an explanation of some of the pharmacological actions of these agents. We shall first discuss the action of iron salts.

1. *The Action of Iron Salts.*

The accelerating action of iron compounds on spontaneous oxidations has long been known and iron is universally regarded as one of the important factors in the oxidations in the cell. Lovén[2] has studied the action of iron on the oxidation of thiolactic acid, and he, Andreasch[3] and Baumann[4] especially have remarked on its extraordinary power of accelerating these oxidations. So far as we can find, however, there are no quantitative experiments on the course of the oxidation. The following experiments in Tables 1 and 2 and in Fig. 1 will show how great is the acceleration produced by small quantities of iron:

[1] Mathews and Walker: This *Journal*, vi, 1909, p. 29.
[2] Lovén: *Journ. f. prakt. Chem.* (2), xxix, 1884, p. 366.
[3] Andreasch: *Monatsh. f. Chem.*, vi, p. 835, 1885.
[4] Baumann: *Zeitschr. f. physiol. Chem.*, viii, 1883, p. 304.

TABLE 1.

March 16, 1908. Acceleration by iron.

TIME SHAKEN MINUTES.	FLASK 1. CYSTEIN + 0.0003 M $FeCl_3$ O_2 ABSORBED IN MM. Hg.	FLASK 2. CYSTEIN + 0.00015 M $FeCl_3$ O_2 ABSORBED IN MM. Hg.	FLASK 3. CYSTEIN ALONE NO IRON. O_2 ABSORBED IN MM. Hg.	TEMPERATURE.	BAROMETER.
10	35	26	1	22.3	749
20	66	50	6	22.4	
30	84	66	8	22.7	
40	110	87	10	22.7	749
60	134	113	15	22.1	748.8

TABLE 2.

Jan. 19, 1909. Effect of small amounts of iron.

TIME SHAKEN MINUTES.	FLASK 1. 0.000006 M $FeCl_3$ AND 0.01 GRAM Na_3AsO_4.	FLASK 2. 0.000006 M $FeCl_3$.	FLASK 3. CYSTEIN ALONE.	TEMPERATURE.
15	6	5	2	19.8
30	16	12	7	20.
60	33	31	19	19.8
90	47	45	29	20.2
175	82	84	58	20.2

This oxidation is one of the best tests for minute amounts of iron. The presence of iron makes itself apparent not only by the acceleration of the oxidation, but by the violet or pink color developed by a ferric salt in a neutral solution of cystein. This test is more delicate than any other we have found for the detection of iron. An amount of iron a little more than $\frac{6}{1{,}000{,}000}$ mol. in concentration is sufficient to double the speed of oxidation of the cystein.

The mechanism of this acceleration undoubtedly involves the formation of an intermediate compound of ferric salt and cystein, this compound being formed and after oxidation of the cystein breaking up again with great speed. This is shown by the fact that the pink color which is due to this intermediate compound, decolorizes instantaneously in the absence of air and that it is

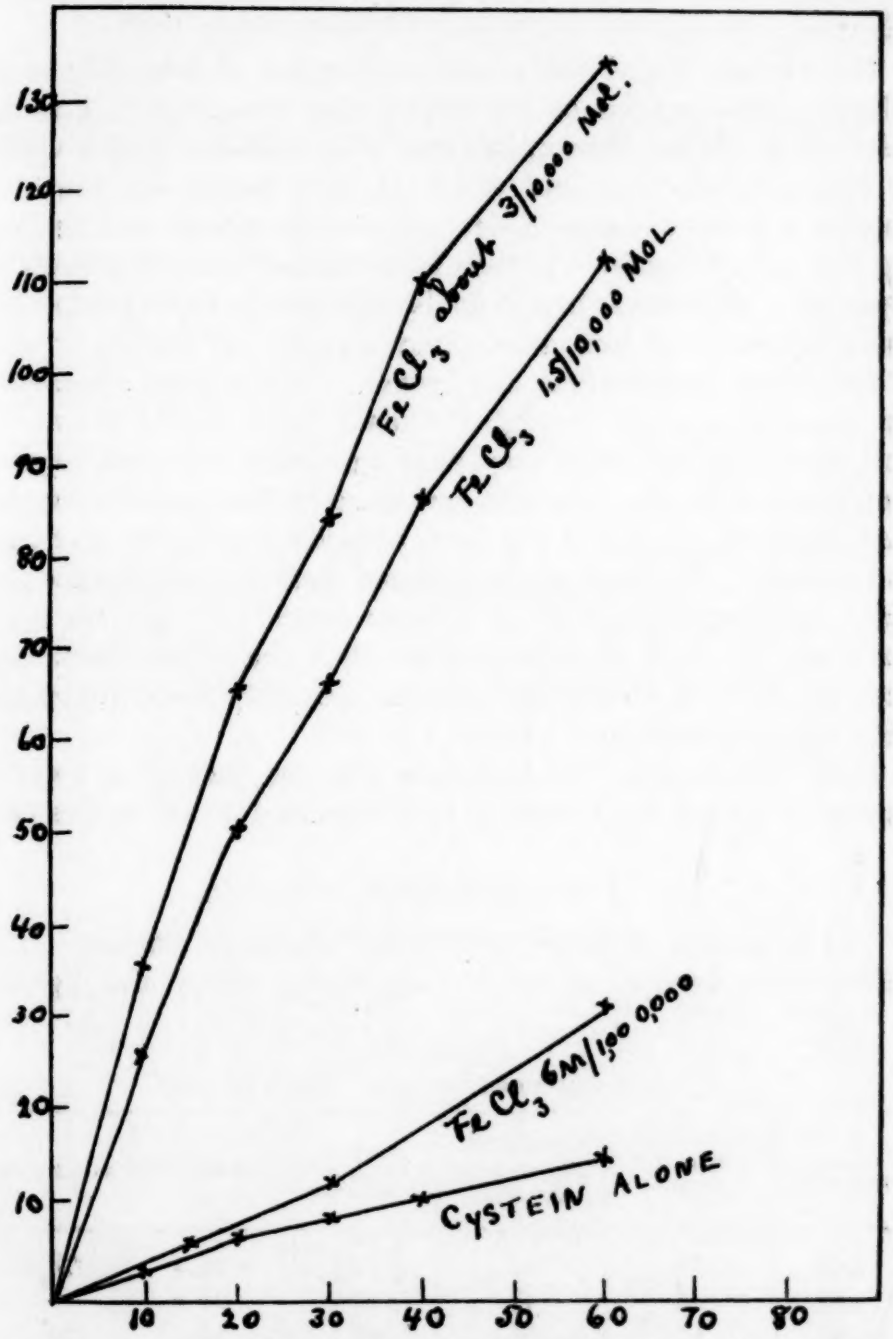

Fig. 1. Comparison of oxidation of cystein alone with cystein in the presence of iron salts. Abscissa = Time in minutes; ordinate = mm. Hg negative pressure in flasks due to absorption of O_2.

formed instantaneously when ferric iron is added to a cystein solution.

The explanation of the accelerating action of iron salts is as follows: The oxygen of the air oxidizes the iron to a ferric salt; this ferric salt then unites with the cystein to form a violet or blue colored compound which at once breaks up, the iron passing a positive charge of electricity to the cystein and becoming ferrous iron again. It then is reoxidized and the process is repeated. We were at first in doubt whether the violet compound was a compound of iron, atmospheric oxygen and cystein or only of ferric iron, hydroxylions and cystein. This question we solved by running hydrogen through the separated solutions of cystein and ferric chloride until they were free from dissolved oxygen and then, while the hydrogen continued to flow and no oxygen was admitted, a little of the ferric chloride was forced over into the cystein. The violet color appeared instantly and at once disappeared; every new addition of ferric chloride brought the color back again. It is therefore certain that the violet compound does not involve atmospheric oxygen, but only ferric iron, probably hydroxylions and cystein.

Iron, therefore, in this oxidation acts the part of an oxygen carrier, as stated by Lovén, as it is supposed to do in the cell.

2. *The Influence of Other Metals.*

The influence of other metals has also been studied. The results may be seen in the accompanying tables and figures.

TABLE 3.[1]

Effects of copper and mercury. March 19, 1908.

TIME, MINUTES.	FLASK 1. CONTROL.	FLASK 2. $\frac{M}{150}$ COPPER ACETATE.	FLASK 3. 0.2 CC. $HgCl_2$ 1 PER CENT.	TEMPERATURE.	BAROMETER.
10	1	12	1	21.4	754.7
33	9	41	13	21.5	
61	17	64	24	22.	754.
82	23	72	33	22.2	753.8

[1] The figures in all the tables represent mm. Hg. negative pressure. developed in the flasks by the absorption of oxygen.

TABLE 4.

Effects of gold, cerium, platinum, thorium and uranium.

TIME, MINUTES.	FLASK 1. 0.01 GM. CERIUM NITRATE.	FLASK 2. 0.01 GM. $AuCl_3$.	FLASK 3. 0.01 GM. $PtCl_4$.	FLASK 4. 0.01 GM. $Th(NO_3)_4$	FLASK 5. 0.01 GM. $UO_2(NO_3)_2$	FLASK 6. CONTROL CYSTEIN.	TEMPERATURE.
15	1	0	0	0	0	4	22.
30	6	0	0	0	1	7	21.8
60	13	0	0	0	4	16	22.
90	18	0	0	0	7	20	22.
Stopped for 16 hours at the end of that time.							
	27	2	2	10	13	30	21.3
Examined the reaction of 2 and 3 and found it acid to alizarin; neutralized to amphoter to litmus with NaOH and shook these two again.							
13		11	7				20.5
28		17	11				22.
63		32	19				22.5
88		44	30				22.5

TABLE 5.

Action of lead, cobalt, zinc and barium. May 7, 1908.

TIME, MINUTES.	FLASK 1. CONTROL CYSTEIN ALONE.	FLASK 2. 0.5 cc. $\frac{M}{3}$ $Pb(NO_3)_2$	FLASK 3. 0.5 cc. $\frac{M}{3}$ $Ni(NO_3)_2$	FLASK 4. 0.5 cc. $\frac{M}{3}$ $Co(NO_3)_2$	FLASK 5. 0.5 cc. $\frac{M}{3}$ $Zn(NO_3)_2$	FLASK 6. 0.5 cc. $\frac{M}{3}$ $Ba(NO_3)_2$	TEMPERATURE 21.6
17	3	0	1	2	0	2	21.8
30	6	0	3	2	0	4	21.8
49	10	0	5	3	0	10	21.8
87	19	0	8	4	0	20	22.0
165	39	0	20	4	0	34	21.9
180	42	0	22	5	0	38	21.9
Not shaken for 16 hours.							
	57	0	33	10	2	53	22.0

TABLE 6.

Action of nickel, zinc, manganese, cobalt and cadmium. July 15, 1908.

TIME, MINUTES.	FLASK 1. $NiCl_2$ $\frac{M}{1000}$	FLASK 2. $ZnSO_4$ $\frac{M}{1000}$	FLASK 3. $MnCl_2$ $\frac{M}{1000}$	FLASK 4. $CoCl_2$ $\frac{M}{1000}$	FLASK 5. $CdCl_2$ $\frac{M}{1000}$	FLASK 6. CONTROL CYSTEIN ALONE.	TEMPERATURE.
20	0	0	1	0	0	1	25.2
40	0	0	3	0	0	4	25.2
60	0	0	6	0	0	7	25.7
101	2	0	12	0	0	15	26.0

TABLE 7.

Action of bismuth and antimony. January 20, 1909. Each flask contained 50 cc. of amphoteric cystein chloride solution containing two grams of cystein hydrochloride.

TIME SHAKEN, MINUTES.	FLASK 1. 0.01 GRAM KSb TARTRATE.	FLASK 2. 0.01 GRAM BISMUTH SUBNITRATE.	FLASK 3. CONTROL CYSTEIN ALONE.	TEMPERATURE.	BAROMETER.
15	1	1	0	21.8	
30	8	8	9	20.5	747.3
45	14?	13	14	20.2	
60	20	18	20	20.	
80	26	24	25	20.1	747.3

Gold, platinum, mercury and copper salts accelerate the reaction when in a neutral solution, not, however, in an acid, but they are somewhat less powerful accelerators than iron. Mercury is far less powerful than copper, which is, however, less active than iron. Cerium nitrate had very little effect; thorium nitrate inhibited strongly, but did not entirely prevent the oxidation; zinc, lead, cobalt and cadmium salts in the strength of solution used completely prevented oxidation for long periods; nickel retarded, but the oxidation was not totally inhibited as by cobalt; manganese and barium salts in $\frac{M}{200}$ solution produced no effect and sodium and potassium salts were inert except in strong solutions.

One of the most interesting features was the difference between cobalt and nickel. When these salts are added to a cystein solution no precipitate is formed, but the red cobalt solution turns to an olive green, while the green nickel solution becomes a deep

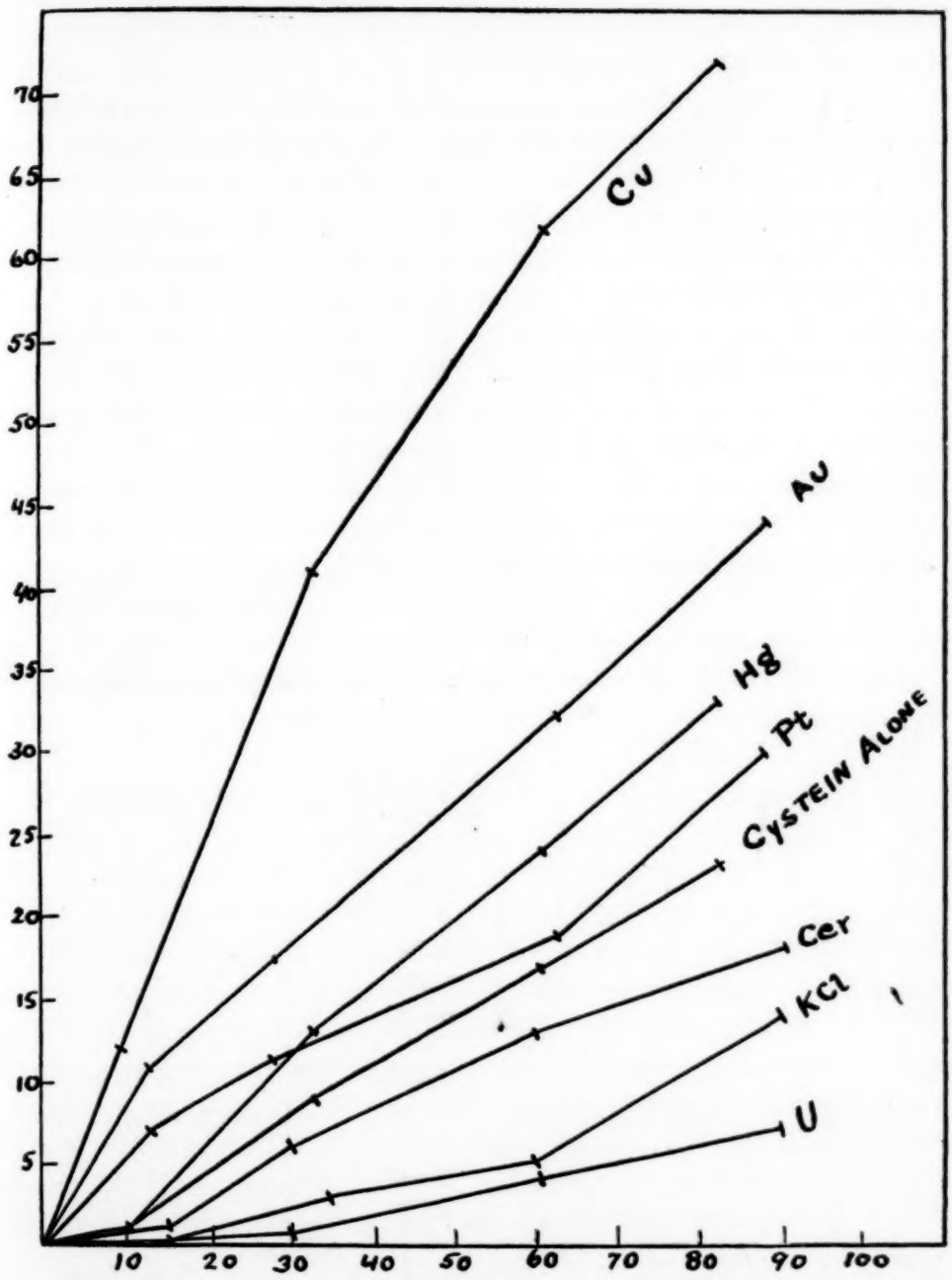

Fig. 2. Comparison of oxidation of cystein alone with cystein in the presence of salts of various metals. Ordinates and abscissas as in Fig. 1. The irregularities in the curves are due to changes in the rate of shaking the flasks and changes in temperature.

port-wine red. Cobalt is more toxic for this oxidation than is nickel. Possibly this is owing to the greater affinity of cobalt for the sulphur of the cystein.

Zinc and lead are particularly toxic and inhibit in very small doses. The behavior of the metals is undoubtedly related to their solution tensions. In Table 8B such a comparison is shown. All the metals below lead, with the possible exception of silver which has not been tried, accelerate the reaction and the acceleration is greatest in the iron and decreases as one goes from iron to copper, mercury and platinum. The next metals above copper, *i. e.*, lead and nickel, strongly inhibit; cadmium and zinc, however, come a little out of the position one would anticipate for them and fall in the class with lead as strong inhibitors. It is interesting to note that in thus coming out of position these metals show the same peculiarity as they do in their toxicity toward *Fundulus* eggs, where cadmium and zinc, were found to be too

TABLE 8 B.

Comparing solution tension of the metals and their action on the spontaneous oxidation of cystein.

METAL.	SOLUTION TENSION IN VOLTS. NORMAL IONIC SOLUTIONS.	ACTION ON THE OXIDATION. DILUTE SOLUTION.
K	3.4?	None
Na	3.0?	"
Ba	2.54	"
Ca	2.28?	"
Mn	0.798	"
Zn	0.493	Inhibits
Cd	0.143	"
Co	−0.045	"
Ni	−0.049	"
Pb	−0.029	"
Fe'''	−0.314?	Accelerates
Cu	−0.606	"
As	−0.570?	"
Hg	−1.027	"
Pt	−1.140?	"
Au	−1.356?	"

toxic and to ally themselves with lead. It is probable that the special toxicity of various metals is due as in this case, to their special affinity for particular constituents of the cell. Above zinc we have manganese, barium, sodium, etc., and these metals in moderate amounts do not appear to affect the rate of oxidation.

The explanation of these facts appears to us to be as follows: The unaccelerated reaction involved is:

(1) $O + \text{cystein} = H_2O + \text{cystin}$.

In the accelerated reaction there is a succession of reactions as:

(2) $O + Me = MeO$.
(3) $MeO + \text{cystein} = \text{cystein MeO}$ (intermediate compound).
(4) $\text{Cystein MeO} = \text{cystin} + Me + H_2O$.

In order that there shall be an acceleration of the reaction by any metal, the time of the several intermediate reactions 2, 3, 4, must be shorter than the time of the reaction 1. To accomplish this the metal must combine rapidly with the sulphur on the one hand and the oxygen on the other, this being the total time required to form the intermediate compound of oxidized metal and cystein. This, however, is but half the reaction. The metal, when in combination, must hand over to the sulphur a positive charge of electricity, be itself reduced and become free again to be reoxidized. The time taken in the first half of the reaction obviously depends on the concentration of the active particles of oxygen, metal and cystein; the time occupied in the second half of the reaction during which the metal remains in combination with the sulphur is very variable with different metals and depends on several factors, the most important of which is the tendency of the metal to give up its positive charge to the sulphur. Only those metals can oxidize the cystein and be themselves reduced which have a sufficient pressure of positive electricity, *i. e.*, a certain ionic potential. These metals are evidently those from ferric iron downward in the scale given in Table 8B. Of these metals those with the highest potential, namely, platinum, gold, etc., must take the least time in the oxidation and hence would oxidize most rapidly and accelerate the reaction most. However, with these metals it happens that owing to their small ionization the first part of the reaction leading to the formation

of the intermediate compound is less rapid than in the case of copper and iron, which ionize more; consequently their total time is greater than in the case of iron and copper. It is probable, also, that the affinity of the reduced metal for sulphur will influence the time the combination of metal and sulphur lasts.

The metals above ferric iron have not sufficient oxidizing potential to oxidize the cystein and consequently do not oxidize it, but they unite with the cystein and remain in combination with the sulphur as shown by Lovén. As they do not easily dissociate again they occupy the place where oxygen ordinarily unites and thus check or prevent the oxidation in the same way

TABLE 8 A.

Effects of Arsenic. January 18, 1909.

TIME, MINUTES.	FLASK 1. 0.01 GRAM. As_2O_3.	FLASK 2. CYSTEIN ALONE	TEMPERATURE.	BAROMETER.
15	2	1	20	754.5
30	8	5	20	
45	16	10	20	
60	19	12	20	
95	35	18	20	754.3
17 hours not shaken. At the end of that time				
	43	27	20	751.0

as the cyanides. The metals of still higher solution tension, such as manganese and the elements above it, dissociate again as rapidly as they unite and accordingly leave the rate unaffected.

3. *The Effect of Arsenic.*

It is well known that arsenic is present in a great many cells and that in minute amounts it acts as a stimulant to the formation of blood, influences the growth of bone and the condition of the skin. It has in some particulars an action like that of iron. In its solution tension, also, it comes not far from iron. It is, therefore, interesting that it accelerates this oxidation, though far less effectively than the iron. This is shown in Table

8A and Fig. 3. Bismuth subnitrate and antimony tartrate did not affect the oxidation in the single experiment tried, using small quantities only.

4. The Effect of Strong Solutions of Neutral Salts.

Strong salt solutions have a powerful effect on the activities of living tissues. They stimulate motor nerves, depress the activity of certain automatic ganglia, stimulate sensory nerves and cause, as an after effect, artificial parthenogenesis in certain

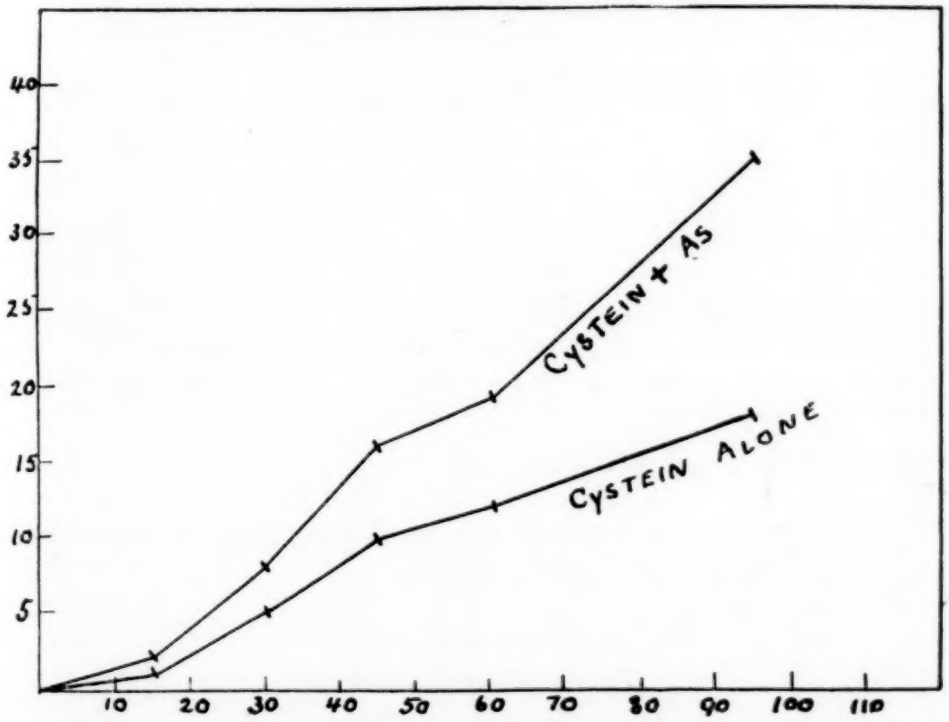

Fig. 3. Effect of sodium arsenate on the oxidation of cystein.

eggs. To produce their effects on eggs Loeb has shown that they must act on the egg when it is in an oxidized condition, and long ago Darwin[1] showed that it was only the oxidized plant cells which could be stimulated by salt solutions. It seems, therefore, not improbable that directly or indirectly such solutions affect the respiratory activities of protoplasm. It seemed worth while, therefore, to see whether this spontaneous oxidation, which shows

[1] Darwin: *Insectivorous Plants*, 2d edition, 1888, p. 214.

so many resemblances to protoplasmic respiration, would be influenced by strong salt solutions. Our experiments have yielded a positive result and we find that strong solutions of sodium and potassium chloride and many other salts strongly retard the oxidation. It is, however, necessary to use solutions as strong as double molecular or stronger, in order to get an effect from some of the salts. The results are shown in the accompanying tables (9, 10 and 11) and in the curves in Fig. 4.

TABLE 9.

Influence of neutral salts.

TIME, MINUTES.	FLASK 1. 5 GRAMS KCl.	FLASK 2. 5 GRAMS Na_2SO_4.	FLASK 3. 5 GRAMS $BaCl_2$.	FLASK 4. 5 GRAMS NH_4Cl.	FLASK 5. 2 GRAMS NaH_2PO_4.	FLASK 6. CONTROL CYSTEIN.	TEMPERATURE.
15	0.5	0	0	1	0*	4	21.1
25	2	4	3	4	0	6	21.1
64	12	14	12	16	0	16	21.4
75	14	16	15	18	0	18	21.3
Not shaken for 15 hours.							
0	36	39	34	36	7	39	21.1
120	63	67	59	62	7	65	21.1

* Retardation in this case due to acidity.

In this experiment the salts were in the concentration of three-fourths to one and one-half molecular.

TABLE 10.

Effects of strong salt solution.

TIME, MINUTES.	FLASK 1. 1 MOL. LiCl	FLASK 2. 4 MOL. KCl	FLASK 3. 4 MOL. NaCl	FLASK 4. SAT. SOL. $BaCl_2$	FLASK 5. 4 MOL. $CaBr_2$	FLASK 6. CONTROL, CYSTEIN ALONE.	TEMPERATURE
15	4	0	2	0	2	4	20.9
35	11	3	8	3	4	11	20.9
60	18	5	11	7	7	16	21.2
105	34	21	22	18	19	28	21.0
212	54	50	46	43	42	50	21.0

Note: This lithium chloride had a trace of iron in it, as was shown by the very faint pink color developed on shaking. We

repeated the experiment with a sample of cystein containing a trace of iron and with recrystallized lithium chloride. (Table 11.)

TABLE 11

Effects of strong salt solution on cystein accelerated by a trace of iron.

TIME, MINUTES.	FLASK 1. 2 MOL. LiCl	FLASK 2. 4 MOL. KCl	FLASK 3. CONTROL, CYSTEIN.	TEMPERATURE.
15	12	8	14	19.8
25	22	11	24	19.8
40	30	14	30	19.2
60	40	18	40	18.9
80	50	28	52	18.8
Machine stopped for a time.				
115	58	36	63	18.0

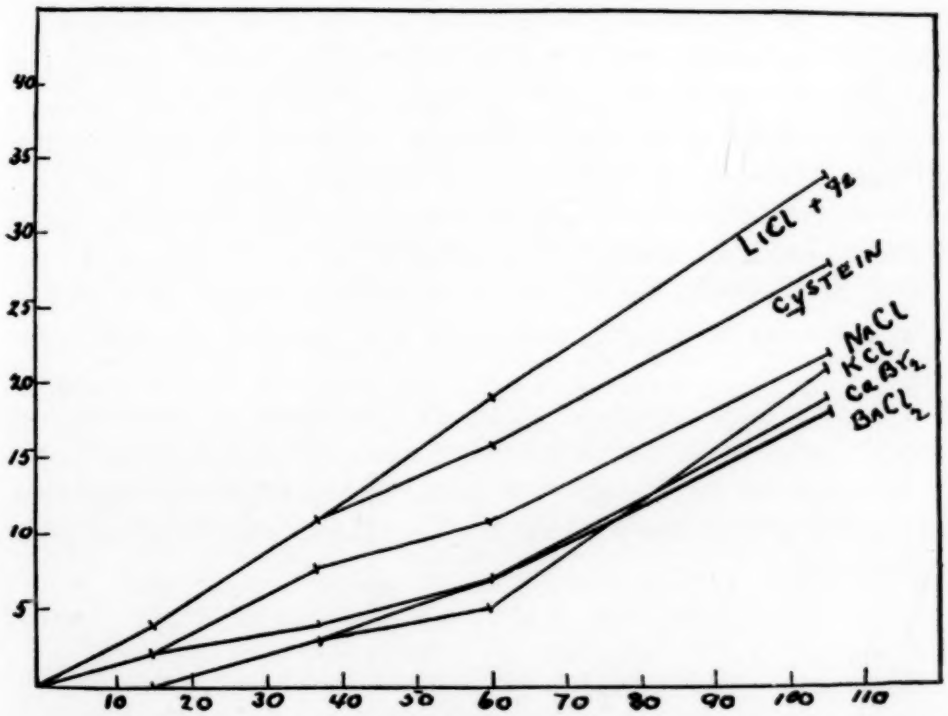

Fig. 4. Effect of strong salt solutions on the oxidation of cystein. Ordinates and absoissas as in other figures.

It is clear from the experiments recorded in Tables 9, 10 and 11, that the addition of considerable quantities of neutral salts such as potassium and sodium chloride strongly retard this oxidation. Potassium chloride appears to act about the same as the sodium and more powerfully than the lithium. Barium chloride and calcium bromide are more effective than the potassium. We are unable at present to explain this action. Possibly the salt interferes with the solution of the oxygen, or possibly it affects the cystein. The fact that neutral salts have this action is of interest.

SUMMARY.

(1) The spontaneous oxidation of cystein to cystin is enormously accelerated by a very small amount of iron. In $\frac{M}{100000}$ solution the oxidation is doubled. The acceleration involves a temporary union of the oxidized iron with the cystein, forming a violet compound. The combination is probably with the sulphur of the cystein.

(2) The oxidation is accelerated also by gold, platinum, copper, and mercury among the metals; and by arsenic among the metalloids. It is strongly retarded by lead, nickel, cobalt, uranium, thorium, zinc and cadmium. Cobalt inhibits more than nickel. These salts inhibit the accelerating action of the iron. The oxidation is not effected by medium amounts of manganese, barium, calcium, sodium or potassium salts. It is greatly reduced by concentrated barium chloride solutions and by 4 molecular solutions of sodium, potassium and calcium chloride solutions.

(3) An explanation is given of the action of the different metals based upon their solution tensions.

(4) In the particulars just noted the spontaneous oxidation of cystein shows many interesting parallelisms to the oxidations of the cells.

ON THE NATURE OF THE CHEMICAL MECHANISM WHICH MAINTAINS THE NEUTRALITY OF THE TISSUES AND TISSUE-FLUIDS.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, May 10, 1909.

It is well-known that the alkali-equivalents of proteins vary markedly with the hydroxyl-concentration of their solutions. Thus 1 gram of serum globulin, in solutions neutral, or approximately so, to litmus ($C_{OH^-} = 10^{-7}$) neutralizes 10×10^{-5} equivalent-gram-molecules of a base, while in solutions neutral to phenolphthalein ($C_{OH^-} = 2 \times 10^{-6}$) 1 gram neutralizes 20×10^{-5} equivalent-gram-molecules of a base.[1] The alkali-equivalent of casein varies very greatly with the hydroxyl-concentration of its solution, 1 gram of casein neutralizing 80×10^{-5} equivalent-gram-molecules of base at neutrality to phenolphthalein and 53×10^{-5} at neutrality to litmus[2] while, as I have recently shown,[3] at "saturation" of the base with casein, that is, at a hydroxyl-concentration of from 10^{-9} to 10^{-8} N, 1 gram of casein neutralizes only 11.4×10^{-5} equivalent gram-molecules of base. I have elsewhere suggested[4] that

[1] W. B. Hardy: *Journ. of Physiol.*, xxxiii, p. 269, 1905.

[2] Soldner: *Landw. Versuchsstat*, xxxv, p. 351, 1888, quoted after Van Slyke and Hart, *Amer. Chem. Journ.*, xxxiii, p. 461, 1905. Courant: *Arch. f. d. ges. Physiol*, l, p. 109, 1891. L. de Jager: *Nederl. Tijdschr. v. Geneesk*, ii, p. 253, 1897, quoted from *Jahresber. f. Thierchem.*, xxvii, p. 276, 1897. H. Timpe: *Arch. f. Hyg.*, xviii, p. 1, 1893, quoted after Raudnitz, *Ergeb. d. Physiol.*, ii. 1, p. 193, 1903. E. Kobrak: *Arch. f. d. ges. Physiol*, lxxx, p. 69, 1900. Laqueur and Sackur: *Beitr. z. chem. Physiol. u. Path.*, iii, p. 193, 1902. Van Slyke and Hart: *Amer. Chem. Journ.*, xxxiii. p. 461, 1905.

[3] T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 469, 1909.

[4] T. Brailsford Robertson: *Journ. of Physical Chem.*, x. p. 524, 1906; xi, p. 453, 1907; xii, p. 473, 1908; this *Journal*, iv, p. 23, 1908; v, p. 147, 1908. T. Brailsford Robertson and Theo. C. Burnett: this *Journal*, vi, p. 105, 1909.

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[3] T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 469, 1909.

[4] T. Brailsford Robertson: *Journ. of Physical Chem.*, x, p. 524, 1906; xi, p. 453, 1907; xii, p. 473, 1908; this *Journal*, iv, p. 23, 1908; v, p. 147, 1908. T. Brailsford Robertson and Theo. C. Burnett: this *Journal*, vi, p. 105, 1909.

the explanation of this remarkable fact lies in the amphoteric, i. e., amino-acid structure of the protein molecule. This structure carries with it the possibility of the formation of double, triple, etc., molecules through the neutralization of the NH_2 groups of single molecules by the COOH groups of others with the extrusion of the elements of water. Regarded from this point of view, the effect of the addition of alkali to a solution of a "saturated" caseinate must consist in the shifting of the equilibrium in the direction of lower complexes, while the addition of acid to a solution of a "basic" caseinate must lead to a shifting of the equilibrium in the direction of higher complexes until, finally, as the addition of acid results in the overstepping of the "saturation" point, the complexes formed assume the properties of matter in mass and a precipitate of free, polymerized casein is formed; such an equilibrium would be of the type:

$$NaXOH + NaXOH \rightleftarrows NaOH + NaXXOH$$

being shifted to the left by increase in the active mass of the sodium hydrate, towards the right by its decrease. This equilibrium would evidently be independent of the total dilution of the system and depend only upon the proportion between the active masses of the sodium hydrate and of the casein. This obviously corresponds with the facts observed.

This phenomenon has, I believe, an intimate bearing upon a very important life-phenomenon; I refer to the practically neutral reaction of all our tissues and tissue-fluids, including, of course, the blood.[1] Since the neutrality of the milieu in which

[1] For literature to 1894 and methods see Hamburger, *Osmotischer Druck und Ionenlehre*, ii, p. 330, 1904. Cf. also H. Friedenthal: *Zeitschr. f. allgem. Physiol.*, i, p. 56, 1902. Hoeber: *Arch. f. d. ges. Physiol.*, xcix, p. 581, 1903; *Physikalische Chemie der Zelle und Gewebe.* Farkas: *Arch. f. d. ges. Physiol.*, xcviii, p. 551, 1903. Fraenkel; *ibid.*, xcvi, p. 601, 1903. Farkas und Scipiades: *ibid.*, xcviii, p. 577, 1903. Pfaundler: *Arch. f. Kinderheilkunde*, xli, p. 174, 1905. Ladislaus von Rhorer: *Arch. f. d. ges. Physiol.*, lxxxvi, p. 586, 1901; cix, p. 391, 1905. Alexander Szili: *ibid.*, cxv, p. 72, 1906. The probable importance of the proteins of the blood in the maintenance of its neutrality was pointed out by Loeb in 1900, *Amer. Journ. of Physiol.*, iii, p. 327, 1900; this possibility had also been previously pointed out by Spiro and Pemsel, *Zeitschr. f. physiol. Chem.*, xxvi, p. 233, 1898.

life-phenomena occur is very general, and disturbance of this neutrality is followed by pronounced pathological phenomena, the mechanism which maintains this neutrality is probably one of extreme importance in the maintenance of life.

The remarkable power which the proteins possess of adjusting their combining-power to the acidity or alkalinity of the medium in which they are dissolved, a power which they owe to their amino-acid structure, suggests at once the mechanism whereby the tissues and tissue-fluids can maintain their neutrality in the presence of varying quantities of CO_2 or of other acid or alkaline waste- or food-products. As we have seen, an increase in the alkalinity of its solution amounting to only .000002 N OH^- (i. e., neutrality to phenolphthalein) in excess of neutrality to litmus increases the alkali-binding power of casein in the proportion of 8 to 5, while a change in hydroxyl-concentration from about $.5 \times 10^{-8}$ N to .000002 N increases the alkali-binding power of casein in the proportion of 8 to 1. Hence changes of relatively enormous magnitude might occur in the CO_2-content of the blood or tissues without appreciably disturbing their neutrality.

Henderson[1] has recently advanced an interesting hypothesis regarding the neutrality of tissue-fluids which, however, differs considerably from that advanced above,—he points out that the rate of change in the alkalinity or acidity of a solution of an acid is a minimum when the dissociation-constant of the acid is equal to the hydrion-concentration at neutrality, and he ascribes the maintenance of the neutrality of the blood to the carbonates and phosphates which it contains. He illustrates his hypothesis by reference to a system tenth-molecular in total carbonic acid and equally concentrated in total phosphoric acid, combined or uncombined with sodium, and he points out that in order to appreciably change the acidity of the system a quantity of acid

[1] Lawrence J. Henderson: *Amer. Journ. of Physiol.*, xv. p. 257, 1906; xxi, pp. 169, 173 and 427, 1908; Lawrence J. Henderson and O. F. Black, *ibid.*, p. 420. In passing I may draw attention to the fact that the possible importance of carbonates in the maintenance of the neutrality of tissue-fluids and the media surrounding living cells was pointed out by Loeb five years ago (J. Loeb, *Arch. f. d. ges. Physiol.*, ci, p. 340, 1904; ciii. p. 503, 1904; *Dynamics of Living Matter*, New York, 1906, p. 95) and by Friedenthal (*Zeitschr. f. allgem. Physiol.* i, p. 56, 1902; iv, p. 44, 1904).

comparable with the total amount of sodium present at neutrality must be added; in order to appreciably increase the alkalinity of the system a comparable amount of free alkali must be added. Similarly, to a mixture of sodium bicarbonate and free carbonic acid which is neutral to litmus an amount of alkali or acid comparable with the total amount of sodium hydrate required to transform the whole of the bicarbonate into carbonate must be added to appreciably affect the neutrality of the solution.

While the mechanism suggested by Henderson must undoubtedly play a part in the preservation of the neutrality of the tissues, yet, I venture to suggest that the proteins must not be overlooked in this connection, since their power of maintaining the neutrality of solutions in which they occur is, as the figures quoted above reveal, extraordinarily great. Thus to 100 cc. of an 8 per cent (= percentage concentration of the proteins in the blood) solution of sodium caseinate neutral to litmus 24 cc. of $\frac{N}{10}$ alkali (or three-eighths of the base originally present) must be added to render the solution neutral to phenolphthalein, or 32 cc. of $\frac{N}{10}$ acid to reach an acidity of .000001 N H^+ while an additional 8 cc. of acid could still be added before all the casein would be precipitated and the solution rendered *just* acid to alizarinsulphonate of sodium, i. e., without attaining an acidity equivalent to .000005 N H^+; moreover to this solution a *further* 26 cc. of $\frac{N}{10}$ acid can be added before all the casein is redissolved and the solution becomes *just* acid to congo red.[1] In all, therefore, to 100 cc. of an 8 per cent solution of casein we can add 66 cc. of $\frac{N}{10}$ acid before the solution attains an acidity corresponding to that of a .00005 N solution of free hydrochloric acid.

Henderson concludes that "the importance of the proteins, through their alkali-compounds in the preservation of neutrality in true solution is surely secondary."[2] He bases this conclusion upon experiments which he has performed upon the difference between the amount of alkali which has to be added to dialysed serum (containing added sodium chloride to hold the proteins in solution) to secure neutrality to rosolic acid and that which has

[1] T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 496, 1909.

[2] Lawrence J. Henderson: *Amer. Journ. of Physiol.*, xxi, p. 446, footnote, 1908.

to be added to secure neutrality to phenolphthalein; he states that "it may be assumed that after the addition of sufficient alkali to make the reaction precisely neutral the only substance present which can still combine with alkalies in appreciable amount are the serum-proteins. Accordingly the power of these solutions to neutralize sodium hydrate may be regarded as a measure of the sodium-protein compounds in the blood-plasma, fibrinogen being disregarded."[1] From what has been said above, however, it will be readily seen that this view is erroneous. Henderson assumes that at absolute neutrality the proteins are uncombined with alkali; as the figures quoted above show, however, this is far from being the case, for at absolute neutrality casein (and the same may be said of serum globulin and, doubtless, of the majority of proteins) is already combined with a considerable quantity of alkali, although upon further addition of alkali casein will bind about one and a half times the quantity of alkali which it can bind at absolute neutrality.

From Henderson's figures it appears that in order to pass from absolute neutrality to neutrality to phenolphthalein about 9 cc. of $\frac{N}{10}$ alkali must be added to 100 cc. of the proteins of serum (8 per cent). Assuming that the behavior of these proteins is analogous to that of casein and that the amount of alkali which they neutralize at absolute neutrality is to that which they neutralize at neutrality to phenolphthalein as 5 is to 8 (the corresponding figure for serum globulin is, according to Hardy, 4 : 8), then the amount of sodium which they must bind at absolute neutrality is equivalent to about 15 cc. of $\frac{N}{10}$ sodium hydrate, i. e., to .015 N sodium hydrate, about fifteen times the estimate of Henderson. The total concentration of sodium in the blood which is not present as sodium chloride cannot be far in excess of .04 N,[2] and this must be present partly in the form of carbonate, partly in the form of bicarbonate and partly in the form of protein-sodium compounds. Hence the part played by these latter, in determining the equilibria and maintaining the neutrality within the blood must be quantitatively comparable with that played by the bicarbonate.

[1] Lawrence J. Henderson: *Amer. Journ. of Physiol.*, xxi, p. 170, 1908.

[2] Cf. Bunge: *Physiological and Pathological Chemistry*, trans. from 4th German edition by F. A. Starling, 1902, p. 263.

The phosphates, at all events in the blood of some animals such as the ox, are present in such minute amount as to be negligible in this connection.[1] Moreover after the total neutralization of the sodium in the blood by acid (= addition of about 40 cc. of $\frac{N}{10}$ acid to 100 cc.) carbonic and phosphoric acids can no longer protect the blood from increase in acidity, but the proteins can still neutralize a considerable quantity of acid; if their power of neutralizing acid is quantitatively comparable with that of casein they can neutralize an additional 62 cc. of $\frac{N}{10}$ acid (to congo red). We may, therefore, I think, conclude that the proteins of the blood play an important part in maintaining its neutrality. In the tissues, of course, where the proportion of protein to bicarbonates, etc. is much greater than it is in the blood, the part played by the proteins in maintaining their neutrality must be proportionally greater.

These phenomena lend considerable support to the view expressed by Sertoli[2] and by Zuntz[3] that the liberation of CO_2 from the blood in the lungs is accompanied by a transfer of sodium from the carbonic acid to the proteins of the plasma. The older view expressed by Bunge was that the liberation of CO_2 is brought about through an interaction between mono-sodium phosphate and sodium bicarbonate:

$$NaH_2PO_4 + NaHCO_3 \rightleftarrows Na_2HPO_4 + H_2O + CO_2$$

the reaction proceeding from left to right in the lungs, where the partial pressure of CO_2 is low, and from right to left in the tissues where the partial pressure of CO_2 is high. The amount of phosphoric acid in the blood is, however, as the above-quoted investigations of Sertoli have shown, too small, at least in many animals, to nearly account for the difference between the CO_2-content of venous and that of arterial blood. If, however, we substitute protein for phosphoric acid in the above reaction we obtain:

$$\text{H-Protein} + NaHCO_3 \rightleftarrows \text{Na-Protein} + H_2O + CO_2$$

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[1] Sertoli: *Hoppe-Seyler's Med. Chem. Unters.*, iii, p. 352, 1868. Mirockowski: *Centralbl. f. d. med. Wissensch.*, 1878, p. 353.

[2] Sertoli: *loc. cit.*

[3] Zuntz: Hermann's *Handbuch der Physiologie*, iv, ii, p. 64.

the reaction proceeding from left to right in the lungs and from right to left in the tissues. We have seen that the proteins of the blood may be assumed to combine, at neutrality, with .015 N sodium which, if it were all converted into sodium bicarbonate, would bind 33.5 vols. per cent of CO_2. The difference between the CO_2-contents of arterial and venous blood is not more than 20 vols. per cent.[1] Hence the quantity of protein in the blood is quite sufficient to account for all of the difference in CO_2-content between the arterial and venous blood. That proteins are able to drive out CO_2 from combination with bases has been shown by Sertoli;[2] casein, especially, when dissolved in solutions of carbonates expels CO_2 and combines with the base to form "basic" caseinate (neutral to phenolphthalein).[3]

SUMMARY

(1) It is pointed out that the power which proteins possess of maintaining the neutrality of solutions in which they occur is very great. Thus to 100 cc. of an 8 per cent solution of sodium caseinate which is neutral to litmus 24 cc. of $\frac{N}{10}$ alkali must be added to render the solution just neutral to phenolphthalein, while 66 cc. of $\frac{N}{10}$ acid must be added to render it acid to congo red, i. e., before the solution attains an acidity corresponding to that of a .00005 N solution of free hydrochloric acid. It is suggested, following the similar suggestions of Spiro and Pemsel and of Loeb, that the proteins of the plasma and tissues play an important part in the maintenance of their neutrality, and it is pointed out that the assumption of Henderson that "the importance of the proteins, through their alkali-compounds in the preservation of neutrality in true solution is surely secondary" is not justified by the facts described in this paper.

(2) It is further suggested, following the similar suggestions of Hoppe-Seyler, Sertoli and Zuntz, that the liberation of CO_2

[1] Cf. various results quoted in Schäfer's *Text-book of Physiology*, article by Pembrey on the "Chemistry of Respiration," i, p. 763, 1898.

[2] Sertoli: *loc cit.*

[3] W. A. Osborne: *Journ. of Physiol.*, xxvii, p. 398, 1901. Van Slyke and Hart: *loc cit.*

from the blood in the lungs is accompanied by a transfer of sodium from the carbonic acid to the proteins of the plasma, the reaction proceeding in the reverse direction in the tissues. It is pointed out that the amount of sodium which is bound by protein in the blood is probably more than sufficient to bind the excess of CO_2 in venous blood over that in arterial blood.

OBSERVATIONS ON URICOLYSIS, WITH PARTICULAR REFERENCE TO THE PATHOGENESIS OF "URIC ACID INFARCTS" IN THE KIDNEY OF THE NEW-BORN.[1]

By H. GIDEON WELLS and HARRY J. CORPER.

(*From the Pathological Laboratory of the University of Chicago.*)

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The fact that deposits of urates are found in the collecting tubules of the kidneys of about one-half of all infants dying during the first two weeks of life, indicates that, in all probability, similar deposits occur frequently in normal infants. Consequently, it must be assumed that such deposits are not often a source of any harm, which assumption accounts for the relatively slight consideration given to these so-called "uric acid infarcts of the new-born." Nevertheless, it seems improbable that deposition in the renal tubules of masses of crystals of ammonium urate can always fail to cause injury, for in the first place these substances are not altogether non-toxic, if we accept the results of the studies of Freudweiler,[2] His,[3] and others who have shown that urates cause local necrosis and act as weak tissue poisons. Furthermore, the very slight solubility of uric acid and the urates insures their remaining in the tubules for some time after they are once deposited; Kaufmann indeed mentioning their persistence for years, finding them in one case in a boy seven years old. Certainly they may serve as the starting point of urate calculi, which occur not infrequently in young children, and it seems not improbable that injury to the renal tissue by these deposits may serve as a starting point for infection. Suppurative pyelitis in infants and young children has been frequently observed, due

[1] This work has been aided by a grant from the Rockefeller Institute for Medical Research.

[2] Freudweiler: *Deutsch. Arch. f. klin. Med.*, lxiii, p. 266, 1899.

[3] His: *Ibid.*, lxvii, p. 81, 1900.

to infection with the colon bacillus as a rule, and no more probable explanation for the localization of the bacilli in the pelvis of the kidney presents itself than that the urate deposits have furnished a *locus minoris resistentiæ*. We have also observed a case which suggests that hemorrhagic infarction of the kidney of the new-born may, at least in some instances, be the result of infection of the pyramids by *B. coli*, presumably localized there for the reasons cited.[1]

Whether actually important as a possible cause of serious harm, as suggested above, or simply a harmless, transitory deposit of urinary constituents in the renal tubules, this process has a considerable interest through its bearing upon the general problems of the physiology and pathology of uric acid metabolism. Many puzzling features offer themselves, the solution of which may throw light on other questions concerning uric acid and its fate. Why are these deposits so common immediately after birth and so uncommon at other times? Why are they observed only in new-born human offspring?[2]

Virchow considered that at the time of birth there resulted a transformation in the entire metabolism on account of the taking in of nourishment, the introduction of direct respiration, and the first exercising of the thermo-regulatory functions, and this manifested itself, among other ways, in an increased elimination of uric acid. Vierordt believed that the feebleness of the oxidation processes during the first days of life was responsible for the excessive output of uric acid, although the direct evidence as to actual oxidizing activity of the new-born human being is not at all clearly in favor of this view. Ebstein thought that in addition to the increased excretion of uric acid another factor was necessary in order to bring about the precipitation of the uric acid in the tubules, and this factor he found in the degenerated renal epithelium, which is injured by the excessive uric acid. Of similar purport is the contention of Flensburg[3] that the tubules

[1] Wells: *Trans. Chicago Path. Soc.*, vii, p. 242, 1909.

[2] The only exception that we have found is the statement by Spiegelberg (*Arch. f. exp. Path. u. Pharm.*, xli, p. 428, 1898) that Pohl observed typical "uric acid infarcts" in the kidney of a monkey which was but a few weeks old.

[3] Inaug. Dissert., Stockholm, 1893, abstracted in Maly's *Jahresber. f. Thierchem.*, xxiii, p. 581, 1893.

of the new-born secrete a hyaline substance which acts as a matrix for urate deposition. Whatever this hyaline matrix is, it would seem to be a colloid forming reversible gels or precipitates, for, as pointed out by Schade,[1] if the colloidal matrix of a urinary precipitate is reversible the precipitate is readily redissolved and does not tend to form calculi, as is the case with the uratic deposits of the new-born; when the colloidal matrix is one which forms non-reversible precipitates, such as fibrin, the deposit is not readily dissolved and forms true concretions.

There is no doubt that the urine of the newly born infant does contain much more uric acid proportionately than the urine of the adult, and this may be ascribed either to excessive formation of uric acid or to a defective destruction. Sjöqvist found that in the new-born the ratio of urea nitrogen to uric acid nitrogen is 74.9 to 7.9, whereas in the adult the ratio is about 85 to 2, and other observers have noticed the relatively large quantity of uric acid in the urine of infants. To account for this increase in uric acid a number of hypotheses have been advanced, most prominent being the view that there is a large and rapid destruction of leucocytes about the time of birth, yielding from their nuclear material the antecedents of the uric acid. We may also imagine that the transformation of the nucleated red corpuscles of the fetus into the non-nucleated corpuscles of the infant gives rise to excessive quantities of free purines. On the other hand a similar result would be obtained if the power of the body to destroy uric acid were decreased, whether by generally defective oxidation as suggested by Vierordt, or from some other more specific cause. In any case the urinary uric acid of the infant must be largely of endogenous origin, for milk is extremely poor in purines.[2]

The fact that the urates are deposited in the collecting tubules may be looked upon as a demonstration of absorption of water in these tubules, since the urates, being relatively insoluble, are precipitated when the dilute urine of the convoluted tubules is concentrated by absorption in the collecting tubules.

[1] Schade: *Münch. med. Wochenschr.*, lvi, p. 3, 1909.

[2] See Orgler: Ueber Harnsäureausscheidung im Säuglingsalter, *Jahrb. f. Kinderheilk.*, lxvii, p. 282, 1908.

Recent studies of purine metabolism have put the matter of uric acid destruction upon a more definite basis than it formerly occupied, and we now know that this destruction is accomplished by definite uricolytic enzymes which have a widespread but irregular occurrence, both as regards various animal tissues and various animal species. For example the bovine kidney is actively uricolytic, while the bovine spleen is devoid of this effect. Similarly we have found, in connection with another study to be published separately, that the spleen of the dog does not destroy uric acid while the liver is very energetically uricolytic. Also there is a difference in the distribution of uricolytic enzymes in the animal kingdom. In an invertebrate, a mollusk, studied by Mendel and Wells[1] it was found that not only was there no uricolytic power, but also that the enzyme, *xanthoöxidase*, which forms uric acid from the oxy-purines, is lacking. The liver of birds will not destroy uric acid perfused through it,[2] as is to be expected from the fact that nitrogenous elimination in the birds is chiefly in the form of uric acid, and in the tissues of the turtle, as an example of a reptile, we have also found complete absence of uricolytic power. On the other hand most mammalian organisms seem to possess uricolytic enzymes, although this is apparently not universal, a fact which will be considered later.

Wiechowski and Wiener[3] give the following as the distribution of uricolytic enzymes in different animal species, according to the collected results of various authors: In bovines, in the kidney, muscles, liver, perhaps in the bone-marrow, but not in the spleen, lung or intestines; in dogs, in the liver but not in the kidneys; in the liver of pigs; in the kidneys and many other organs of the horse; in the liver but not in the kidneys of rabbits. To this list we may add, from our own observations, that the liver of the guinea-pig is actively uricolytic, that the liver and the other viscera of the turtle seem to be entirely inactive, and that the spleen, bone-marrow and probably the leucocytes of the dog are not uricolytic.

[1] Mendel and Wells: *Amer. Journ. of Physiol.*, xxiv, p. 170, 1909.

[2] Friedman and Mandel: *Arch. f. exp. Path. u. Pharm.*, Supplementband, 1908, p. 199.

[3] *Beitr. z. chem. Physiol. u. Pathol.*, ix, p. 247, 1907.

The activity of this enzyme is very considerable; beef kidney, for example, will totally destroy the uric acid added to it, if not too great in amount, in from four to seven hours.[1] However, in this respect it stands behind the remarkable rapidity of some of the other enzymes concerned in purine metabolism, for Schittenhelm[2] has observed quantitative conversion of guanine into uric acid by bovine spleen in one to two hours.

Mendel and Mitchell[3] have studied the development of the enzymes of purine metabolism in the developing pig embryo, with the following results: Nuclease appears at an early period, the power to free purines from the tissues during autolysis being present in 50 mm. embryos, the smallest studied. Adenase is present at the same early stage, but xantho-oxidase does not manifest itself in the livers of fetal pigs of 200 mm. length, although present in the liver of sucking pigs. Likewise uricolytic enzymes were not present in 200 mm. embryos, and only slight uricolytic power was demonstrable in the liver of sucking pigs, about two months old, although the liver of adult pigs is actively uricolytic.

These results obtained in a typically omnivorous animal, the pig, if transcribed to another omnivorous animal, man, would seem to offer a ready explanation of the high uric acid content of the infant's urine and the resulting uric acid infarcts in the kidneys. They suggest that during fetal life any necessary uricolysis is performed for the fetus by the mother, either in the placenta or after absorption of the uric acid of the fetal circulation into the maternal blood. After birth the uricolytic power, which does not appear until about this time, is more or less imperfectly developed for a few days or weeks, during which time uric acid that should be destroyed is excreted in the urine. In support of this theorization are the results obtained by Spiegelberg[4] with dogs. He saturated the tissues of dogs, both young and adult, with uric acid by subcutaneous injection, and found that the power of young dogs to destroy uric acid was much less

[1] Künzel and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, v. p. 389, 1908.

[2] Schittenhelm: *Zeitschr. f. physiol. Chem.*, lvi, p. 21, 1908.

[3] Mendel and Mitchell: *Amer. Journ. of Physiol.*, xx, p. 97, 1907.

[4] Spiegelberg: *Arch. f. exp. Path. u. Pharm.*, xli, p. 428, 1898.

than that of old dogs. For example, when 0.1 gram of uric acid per kilo of body weight was injected into puppies, 53 per cent could be recovered in the urine, while with adult dogs under the same condition but 5.6 per cent was excreted. Also the urine of puppies becomes turbid with urates, and uratic infarcts of typical appearance are produced in the kidneys when but 0.05 to 0.1 gram uric acid per kilo is injected, whereas much larger quantities of uric acid cause no similar effects in adult dogs. Therefore it would seem that in young dogs the power to destroy uric acid is much less than in adult dogs, the possibility that the turbidity of the urine and the renal infarcts are due to poor solvent action of the urine in the young animal having been excluded.

With the object of testing this hypothesis, namely, that the high uric acid excretion and the occurrence of uric acid infarcts of young infants is due to the failure of development of uricolytic enzymes until after birth, as in the pig, a series of experiments was undertaken. The only similar experiments recorded in the literature are those of Schittenhelm and Schmidt,[1] which were as follows:

1. To the ground tissue of the kidneys (25 grams) of a full-term child, 0.1 gram uric acid was added, and autolysis was permitted for 4 days with frequent shaking for the purpose of aeration. At the end of this time no uric acid could be recovered.

2. 0.1 gram uric acid added to the extract of the kidneys (31 grams) of a full-term infant, under the same conditions. 0.018 gram uric acid recovered.

3. 140 grams of liver extract of an infant (age not stated) autolyzed with 0.3 gram of uric acid, a current of air being conducted through the mixture for seven days. No uric acid recovered.

4. 82 grams ground muscle from a seven-months' fetus and 0.2 gram uric acid autolyzed five days, with frequent shaking. No uric acid recovered.

5. 70 grams ground muscle from a full-term infant autolyzed with 0.2 gram uric acid for 14 days, frequently shaken. No uric acid recovered.

6. 58 grams of lung extract from a full-term fetus were autolyzed with 0,15 gram uric acid for 14 days, frequently shaken, 0.05 gram uric acid was recovered.

They further state that "We can add to these results that we obtained a questionable result with intestine emulsion. Unfor-

[1] *Zeitcshr. f. exp. Path. u. Ther.*, iv, p. 424, 1907.

tunately, material was lacking to repeat the experiments with lung and intestine. In any case these two organs, even if they possess any uricolytic power, stand much below the kidney, liver and muscle in activity. These three organs possess a very intensively acting uricolytic enzyme, agreeing with the organs of mammalian animals in which it is also found that the liver, kidney and muscle accomplish the destruction of uric acid."

These experiments would seem at once to give a negative answer to the possibility of explaining the conditions of uricolysis in the human infant on the basis of the results obtained by Mendel and Mitchell with pig embryos, for they indicate that the organs of the human fetus at term are able to destroy uric acid with great activity. However, there still remained the possibility that this uricolytic power develops late in fetal life, and that it is not always present at birth, and also, because of the unexpected result of these experiments of Schittenhelm and Schmidt, it seemed desirable to repeat them, and also to study embryos of different ages to ascertain the time at which the uricolytic power appears. It was not until we had already begun upon the work that we became acquainted with a later paper from Schittenhelm[1] which made the positive results of Schittenhelm and Schmidt seem even more surprising, and repetition more necessary. In this later paper, dealing with purine metabolism in adult man, it is reported that human liver, although actively oxidizing purines to uric acid, has little if any uricolytic power. They were not able to obtain satisfactory results from human kidneys. Human intestine was found to transform guanine into xanthine. They also state that, in a communication at that time unpublished, Wiechowski had likewise reported that he was unable to demonstrate any considerable evidence of uricolysis in human tissues, that he could find no allantoin in the urine after subcutaneous injection of uric acid, and that as 60 to 80 per cent of the uric acid reappeared in the urine he concluded that uric acid is not attacked by human tissues.[2]

[1] Künzel and Schittenhelm: *Zentralb. f. Physiol. u. Pathol. d. Stoffwechsels*, iii, p. 721, 1908.

[2] This article has since been published, *Arch. f. exp. Path. u. Pharm.*, lx, p. 185, 1909.

It would seem strange indeed that the new-born infant should have a well-developed power to destroy uric acid, as Schittenhelm and Schmidt maintain, while the adult has lost this capacity, yet Schittenhelm does not seem to consider this discrepancy. In one of his most recent articles[1] he refers to the previous observations on uric acid destruction by the tissues of the new-born, and considers them as conclusively proving that such human tissues are actively uricolytic.

The results of our own experiments, which are recounted in detail below, seem to be quite conclusive in showing that adult human tissues have no distinct power to destroy uric acid *in vitro*, thus agreeing with Künzel and Schittenhelm and with Wiechowski; furthermore, with fetal tissues we obtain results entirely the opposite from the paradoxical findings of Schittenhelm and Schmidt, for we find the human fetus at all stages of its development is devoid of any appreciable uricolytic power in its chief tissues and organs. Our method of experimentation was as follows:

The organ or tissue to be examined was ground fine in a hashing machine, placed in three volumes of toluol water and let autolyze at room temperature over night, then strained through cheese cloth. To the emulsion was then added the uric acid, which had been dissolved in boiling water by adding, drop by drop, just enough dilute sodium hydroxide to complete solution, and then quickly cooled. The mixture was then allowed to autolyze in a thermostat at about 37° in the presence of abundant toluol, a current of air being drawn through. After 24 or 48 hours, usually, the autolysis was stopped by boiling the mixture, coagulated with a minimum of acetic acid, filtered, and the residue well washed by being boiled with fresh water, alkalinized faintly with sodium carbonate to assure solution of all residual uric acid, reacidified and refiltered and washed. In the filtrate the uric acid is precipitated by the copper sulphate and sodium bisulphite method of Krüger and Solomon, filtered, washed, decomposed with hydrogen sulphide, filtered, concentrated to 50 cc. in the presence of hydrochloric acid, and weighed directly after filtering off in a weighed Gooch crucible. When the precipitate gave any indication of any impurity it was repurified by

[1] Brugsch and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, v, p. 406, 1908.

precipitating from the solution in concentrated sulphuric acid. After making the usual correction for solubility, control experiments with boiled tissue extracts showed that from 85 to 95 per cent of the added uric acid can be recovered by this method, the losses probably being partly due to retention in the precipitates, partly to losses in the copper sulphate precipitation and decomposition, and partly, perhaps, to the destructive action of the alkali used in dissolving the uric acid. These slight losses are, however, of little significance, for we have always found that when a tissue has any uricolytic power at all this is always capable of destroying all the uric acid present under the conditions of these experiments. Therefore the isolation and weighing of the uric acid is really little more than a control procedure, for when tissues which are uricolytic are used there is no precipitate whatever when the copper sulphate is added to the filtrate, in marked contrast to the voluminous precipitate produced in the filtrate when there has been no uricolysis.

A. Uricolysis by Adult Human Tissues.

I. Tissues from the body of a man, aged 40, who died suddenly of heart disease. Twelve hours after death the organs, showing no visible pathological changes, were treated as above, 100 grams of tissue being used with each, the spleen, kidney, and liver, and 75 grams of muscle tissue. Autolysis continued 48 hours with a current of air passing through the autolyzing mixture. From all the uric acid was recovered in large amounts, as follows:

TISSUE.	URIC ACID ADDED.	URIC ACID RECOVERED.	PER CENT RECOVERED.
	grams.	*grams.*	
Spleen	0.1794	0.160	89
Kidney	0.1640	0.135	82
Liver	0.1700	0.185	109*
Muscle	0.1740	0.1315	76

*Presumably this slight gain in uric acid depends upon the power of human liver to form uric acid from xanthine present in autolyzing extracts.

II. Tissues from body of a healthy man, aged 22, dying from a stab wound, and autopsied almost immediately after death. 100 grams of tissue used for each experiment, including one with liver extract heated to 100° for fifteen minutes. Autolysis with air current for 48 hours. Results:

TISSUE.	URIC ACID ADDED.	URIC ACID RECOVERED.	PER CENT RECOVERED.
	grams.	*grams.*	
Kidney	0.1258	0.1102	87
Spleen	0.1186	0.0958	81*
Liver (fresh)	0.1953	0.1833	93
Liver (boiled)	0.1019	0.0988	97

*Result of this analysis uncertain, as there was some slight loss.

B. Experiments with Fetal Tissues.

III. Female fetus, weight 727 grams, length 34 cm., therefore presumably about the sixth or seventh month. Liver weighed 34 grams, and was allowed to autolyze with 0.1896 gram uric acid for 48 hours with constant air current. Recovered 0.1821 gram uric acid, or 96 per cent; therefore no uricolysis.

IV. A fetus weighing 105 grams, length 16 cm., therefore probably about the fourth month. This was permitted to autolyze 24 hours with uric acid (with air current) and for control 50 grams of dog liver and 85 grams of guinea-pig liver (5 livers) treated in the same way with the following results:

TISSUE.	URIC ACID ADDED.	URIC ACID RECOVERED.	PER CENT RECOVERED.
	grams.	*grams.*	
Fetus	0.1136	0.0834	73.4
Dog liver	0.1060	0.0	0
Guinea-pig liver	0.1319	0.0	0

V. Fetus, length 23 cm., weight 306 grams, presumably about the fifth month. Entire fetus ground up and extracted in the

usual way. Extract divided into two equal parts. Autolysis for 48 hours in air current at 37°, with following results:

(1). 150 grams tissue—added 0.1445 gram uric acid. Recovered 0.1393 gram uric acid or 96 per cent.

(2). 150 grams tissue—added 0.1405 gram xanthine. Recovered 0.1407 gram xanthine.

The recovery of as much xanthine as was added, without making any correction for loss by solubility or in manipulation, shows the absence of xantho-oxidase, and also suggests that some xanthine may have been formed from the tissue purines under the conditions of the experiment. Other experiments, to be reported later, show that from a very early stage the human fetus has the power of forming xanthine from guanine.

VI. Full term fetus, well developed, weight 3400 grams. Apparently died from asphyxiation because of podalic delivery without attendance. Made extracts of 150 grams of mixed viscera (including spleen, lungs, thymus, adrenals, pancreas and gastro-intestinal tract), 143 grams of muscle and 24 grams of kidneys, and let the three extracts autolyze with uric acid 48 hours in an air current. On account of a back-flow of the material from one flask into another of the series the results with each tissue cannot be distinguished, but as to the three lots 0.372 gram of uric acid had been added and the total amount recovered was 0.347 gram or 93 per cent; it is evident that there was no destruction of uric acid by any of these tissues.

VII. Full term fetus, living but three hours on account of congenital heart defect. Experiment performed in the usual manner.

TISSUE.	MATERIAL ADDED.	MATERIAL RECOVERED.	PER CENT RECOVERED.
grams.	*grams.*	*grams.*	
1. Liver (100)...	0.1514 uric acid	0.2002 uric acid	132
2. Liver (100)...	0.0958 xanthine	0.0856 uric acid	
3. Viscera (95). .	0.1514 uric acid	0.1706 uric acid	112
4. Viscera (95). .	0.0958 xanthine	0.0463 uric acid 0.0687 xanthine	
5. Kidneys (22) .	0.1514 uric acid	0.1488 uric acid	98

This experiment demonstrates at one and the same time the presence of xantho-oxidase and the absence of uricolytic enzymes in the tissues of the full term fetus. The xantho-oxidase of the liver has converted the xanthine which was added into uric acid, which is then not destroyed, and it has also converted the purines present in the autolyzing liver tissue into uric acid. With the mixed viscera the conversion of xanthine into uric acid has not been so complete, possibly because of the fact, pointed out by Schittenhelm, that the enzymes of one organ may inhibit or destroy the oxidases of another organ. The time of development of xantho-oxidase in the human fetus will be more fully discussed in a later paper.

C. Uricolytic Action of Placenta.

As Wiechowski[1] in particular has pointed out, the animals which destroy uric acid excrete allantoin in their urine as a product of this uricolysis. The older literature states that allantoin may be found in the urine of pregnant women (Gusserow) and of infants during the first few days after birth, but apparently it is not ordinarily present in the urine of adults. As we have found that the tissues of neither infants nor adults destroy uric acid, and hence presumably produce no allantoin, the possibility presented itself that the placenta might have uricolytic power, an assumption which fits in perfectly with the classical finding of allantoin in the amniotic fluid (cow) as well as with its reputed presence in the urine of pregnant women. Our experiments, however, showed absolutely no evidence of uricolytic power in the human placenta. Two fresh human placentas were obtained, ground up, and 250 grams used for each of the following experiments:

TISSUE.	URIC ACID ADDED.	URIC ACID RECOVERED	PER CENT RECOVERED.
	grams.	*grams.*	
1. Placenta (boiled).....	0.1427	0.1372	96
2. Placenta (fresh).....	0.1513	0.1467	97
3. Placenta (fresh).....	0.1486	0.1399	94

[1] *Arch. f. exp. Path. u. Pharm.*, lx, p. 185, 1909.

1 and 2 represented the extract from one placenta; 3 was from a different placenta.

The failure to demonstrate the presence of uricolytic power in human placenta as well as in other human tissues, causes some question to arise as to the validity of the claims that allantoin may be present in the urine of pregnant women. Wiechowski, in the article cited above, discusses these claims. He could find no authority whatever for the current statements that human amniotic fluid and the urine of infants during the first eight days of life contain allantoin, and he finds reason to doubt the methods by which allantoin is supposed to have been demonstrated in human urine. The negative result of studies of uricolysis by human tissues, including placenta, affords still further ground for skepticism regarding the occurrence of allantoin in the urine during pregnancy, and makes necessary a re-investigation of this question by more modern methods.

D. Influence of Serum upon Uricolysis.

It having been demonstrated in many ways that fresh blood serum exercises an inhibiting effect upon the ordinary processes of autolysis, and as uricolysis may be considered as an analogous process to the extent that it is a destruction of organic compounds by intracellular enzymes, it seemed possible that the negative results obtained in these experiments might perhaps be dependent in some measure upon an anti-uricolytic action of the serum present in the tissue extracts. An experiment was performed to decide this question, with negative results.

Fresh dog liver, weighing 250 grams, was ground up and let stand over night in toluol water at room temperature. Strained through cloth, made up to 400 cc., and divided into four 100 cc. portions. These were then let autolyze for 17 hours at 37° with constant air current, with the following additions:

1. Added 200 cc. water and 0.0966 gram uric acid. Result —No uric acid could be recovered.

2. Added 200 cc. water, then heated to 100° for 15 minutes, cooled, and added 0.1034 gram uric acid. Result—Recovered 0.0833 gram uric acid, or 86 per cent.

3. Added 200 cc. dog *serum*, freshly obtained, and 0.1175 gram uric acid. Result—No uric acid could be recovered.

4. Added 200 cc. of serum that had been heated at 75° for 20 minutes, and 0.0848 gram uric acid. Result—No uric acid could be recovered.

This experiment shows the power of dog liver to quantitatively destroy uric acid in a much shorter time than our experiments were continued with human tissues, and indicates that dog serum does not check the uricolytic power under the conditions of the experiment. It is possible that a much larger quantity of serum might inhibit uricolysis, or that the rate of uricolysis is slowed during the few hours needed for its completion, but it was not considered advisable to follow up this matter at this time, since the results of this experiment indicate that the quantity of serum present in the tissues with which we were working can not account for the total lack of uricolytic activity observed. The fact that in the living dog when the uric acid is parenterally injected it is rapidly destroyed by tissues that are bathed in constantly changing serum, makes it improbable, *a priori*, that dog serum can inhibit uricolysis by dog's tissues to any great extent. We have not attempted to learn whether human tissues washed free from serum have any more power to produce uricolysis than tissues in the presence of such small amounts of serum as are admixed with the organ after its removal from the body—that is, to finally establish that the failure of uricolysis by human tissues is due to a lack of uricolytic enzymes, as seems probable, rather than to any highly developed anti-uricolytic property present in human serum and absent in dog serum.

E. Uricolytic Action of Leucocytes of Dog.

Earlier investigators of gout and uric acid have sought for a uricolytic action on the part of the blood, with negative results. Thinking that possibly the leucocytes of the dog, in which animal we have very active uricolytic enzymes in the liver, might possess uricolytic power, and having already demonstrated the absence of uricolysis in the spleen of the dog, an experiment with leucocytes and bone-marrow was performed.

A large adult dog received injections of an emulsion of 15 grams aleuronat, 5 cc. turpentine, and 150 cc. water, one-third into the pleura and two-thirds into the peritoneum. Killed

after 18 hours, and 500 cc. turbid, blood-stained exudate removed from the peritoneum, but none was obtained from the pleura. Left the fluid standing over night on ice, drew off the supernatant serum and obtained a sediment of 60 cc. of leucocytes mixed with red corpuscles and a little serum. The ribs of several dogs were removed, cleaned free from adherent tissue, ground up, extracted over night in toluol water, and the resulting emulsions of marrow tissue strained through cheese cloth. The leucocytes and the marrow extract were allowed to act upon uric acid in the usual way for 48 hours, and found not to destroy it. There being somewhat more than the usual loss of uric acid in the marrow extract this experiment was repeated twice with similar results, although in the last experiment the result was not different from that frequently obtained with other non-uricolytic tissues.

TISSUES.	URIC ACID ADDED.	URIC ACID RECOVERED.	PER CENT RECOVERED.
	grams.	*grams.*	
1. Leucocytes	0.0795	0.0716	90
2. Marrow	0.1590	0.1129	71
3. Marrow	0.1580	0.1146	73
4. Marrow	0.1543	0.1317	85

CONCLUSIONS.

From the foregoing experiments, together with recent observations by other investigators, it seems well established that the tissues of the adult human being possess no uricolytic enzymes, or at least none capable of producing appreciable uricolysis under conditions that give total destruction of uric acid by tissues from many other mammals. If further studies including all the tissues of the human body show a total absence of uricolytic power, which seems probable from the *in vivo* experiments of Wiechowski, our present conceptions of gout and purine metabolism must be much modified. The tissues of the human fetus at various stages of development show no more evidence of uricolysis than do the tissues of the adult. It is impossible to explain the positive results reported by Schittenhelm and Schmidt

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This experiment shows the power of dog liver to quantitatively destroy uric acid in a much shorter time than our experiments were continued with human tissues, and indicates that dog serum does not check the uricolytic power under the conditions of the experiment. It is possible that a much larger quantity of serum might inhibit uricolysis, or that the rate of uricolysis is slowed during the few hours needed for its completion, but it was not considered advisable to follow up this matter at this time, since the results of this experiment indicate that the quantity of serum present in the tissues with which we were working can not account for the total lack of uricolytic activity observed. The fact that in the living dog when the uric acid is parenterally injected it is rapidly destroyed by tissues that are bathed in constantly changing serum, makes it improbable, *a priori*, that dog serum can inhibit uricolysis by dog's tissues to any great extent. We have not attempted to learn whether human tissues washed free from serum have any more power to produce uricolysis than tissues in the presence of such small amounts of serum as are admixed with the organ after its removal from the body—that is, to finally establish that the failure of uricolysis by human tissues is due to a lack of uricolytic enzymes, as seems probable, rather than to any highly developed anti-uricolytic property present in human serum and absent in dog serum.

E. Uricolytic Action of Leucocytes of Dog.

Earlier investigators of gout and uric acid have sought for a uricolytic action on the part of the blood, with negative results. Thinking that possibly the leucocytes of the dog, in which animal we have very active uricolytic enzymes in the liver, might possess uricolytic power, and having already demonstrated the absence of uricolysis in the spleen of the dog, an experiment with leucocytes and bone-marrow was performed.

A large adult dog received injections of an emulsion of 15 grams aleuronat, 5 cc. turpentine, and 150 cc. water, one-third into the pleura and two-thirds into the peritoneum. Killed

after 18 hours, and 500 cc. turbid, blood-stained exudate removed from the peritoneum, but none was obtained from the pleura. Left the fluid standing over night on ice, drew off the supernatant serum and obtained a sediment of 60 cc. of leucocytes mixed with red corpuscles and a little serum. The ribs of several dogs were removed, cleaned free from adherent tissue, ground up, extracted over night in toluol water, and the resulting emulsions of marrow tissue strained through cheese cloth. The leucocytes and the marrow extract were allowed to act upon uric acid in the usual way for 48 hours, and found not to destroy it. There being somewhat more than the usual loss of uric acid in the marrow extract this experiment was repeated twice with similar results, although in the last experiment the result was not different from that frequently obtained with other non-uricolytic tissues.

TISSUES.	URIC ACID ADDED.	URIC ACID RECOVERED.	PER CENT RECOVERED.
	grams.	*grams.*	
1. Leucocytes	0.0795	0.0716	90
2. Marrow	0.1590	0.1129	71
3. Marrow	0.1580	0.1146	73
4. Marrow	0.1543	0.1317	85

CONCLUSIONS.

From the foregoing experiments, together with recent observations by other investigators, it seems well established that the tissues of the adult human being possess no uricolytic enzymes, or at least none capable of producing appreciable uricolysis under conditions that give total destruction of uric acid by tissues from many other mammals. If further studies including all the tissues of the human body show a total absence of uricolytic power, which seems probable from the *in vivo* experiments of Wiechowski, our present conceptions of gout and purine metabolism must be much modified. The tissues of the human fetus at various stages of development show no more evidence of uricolysis than do the tissues of the adult. It is impossible to explain the positive results reported by Schittenhelm and Schmidt

with the tissues of the infants at or near term, but in view of the unanimity of opinion as to the absence of uricolytic activity in adult tissues, their results are *a priori* doubtful. We cannot, therefore, explain the presence of urate deposits in the kidney of new-born infants as due to tardy development of uricolytic enzymes in the human fetus, as suggested by the results obtained by Mendel and Mitchell with pig embryos. More probably the cause of these deposits is simply the high proportion of uric acid in the urine of the new-born, as maintained by Sjöqvist, the deposition perhaps being favored by local conditions in the kidney, such as the hyaline matrix described by Flensburg. Our failure to find evidence of uricolytic activity in either fetus or placenta, together with the lack of uricolytic enzymes in human organs and tissues, throws doubt upon the statements in the older literature that allantoin is found in the urine of pregnant women and new-born infants, since so far as known allantoin is formed only through decomposition of uric acid.

As additional observations on the distribution and behavior of uricolytic enzymes, may be mentioned the finding of active uricolytic power in the liver of the guinea-pig; its absence in the organs of the turtle; its absence in the spleen, and probably also in the bone-marrow and leucocytes of the dog; and also the failure to demonstrate inhibition of uricolysis by serum (dog).

PROTEIN METABOLISM IN CYSTINURIA. II.

By HORATIO B. WILLIAMS and CHARLES G. L. WOLF.

(From the Departments of Chemistry and Physiology, Cornell University Medical College, New York.)

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During the last five years, four papers have appeared, detailing the results of very careful and complete analyses of the urine in cases of cystinuria.[1]

The conclusions reached from these recent investigations are at variance on certain important points, and it has been suggested that the explanation may lie in individual peculiarities of the cystinurics. To decide this question requires the accumulation of data from the study of as many cases as possible, and it was for the purpose of adding to existing evidence that the work herein reported was undertaken.

The questions which so far have not been definitely settled, and are at present under discussion are:

(1) The relation of cystin excretion to the amount of protein food ingested.

(2) The ability of the patient to oxidize cystin administered as such *per os*.

(3) The ability of the patient to deamidate other amino-acids.

(4) The occurrence of diamines in the urine.

(5) The occurrence of other amino-acids, such as leucin and tyrosin in the urine.

(6) Whether the increase in neutral sulfur which forms so notable a feature of the sulfur partition in cases of cystinuria is due entirely to cystin.

The material which forms the basis of this communication

[1] Loewy and Neuberg: *Zeitschr. f. physiol. Chem.*, xliii, p. 338, 1904. Alsberg and Folin: *Amer. Journ. of Physiol.*, xiv, p. 54, 1905. Loewy and Neuberg: *Biochem. Zeitschr.*, ii, p. 213, 1906. Wolf and Shaffer: this *Journal*, iv, p. 439, 1908.

is from a single case, but fortunately the patient was a man of intelligence and sufficiently interested to observe the necessary care in the collection and preservation of the twenty-four hour specimens of the urine.

We also had his cooperation in taking the various substances that were administered by way of experiment.

A brief abstract of the clinical history follows:

J. M., æt. 29, single, German, resident during the last thirteen years in the United States. Occupation, clerk.

Family history negative, except that five maternal uncles suffered with rheumatism, sciatica or lumbago.

Patient has had pneumonia and measles. Muscular rheumatism at intervals since æt. 18. Specific urethritis at 21, with several recurrences. Lues at 25. During the last four years has had a great deal of gastric indigestion, so severe at one time as to keep him in hospital for a week (about 8 months ago). His weight has varied from 115 to 134 and is at present 129 pounds.

About a year and a half ago he noticed after urination a little lump in the meatus, and rolled it out. It was light yellow in color, smooth and rather hard, but he was able to crush it with his fingers. He did not preserve it. About the last of October, 1908, he passed a similar substance in much the same way.

He has had very little trouble with his stomach of late, but has been very careful in the matter of diet. During the last ten days he has had a great deal of pain in the back, referred especially to the region of the scapulæ. It is for this condition that he presents himself.

Physical examination reveals nothing significant, except a chronic prostatitis and urethritis. The urine contains numerous shreds and a white sugary-looking sediment, which settles rapidly, is insoluble in hot water, or in acetic acid, but readily soluble in ammonia, especially with gentle heat, and recrystallizes on evaporation of the ammonia. Microscopically, the sediment is seen to consist of colorless hexagonal plates.

The sediment manifests a characteristic tendency to cling to the sides of the glass container when the urine is poured off.

Methods.

Because of the lack of an accurate method of determining cystin, which could be applied with a reasonable amount of labor to the estimation in a number of urines, the amount of cystin has usually been calculated from the excess of neutral sulfur over the amount found in normal urines, assuming that this excess is due entirely to cystin.

Gaskell[1] has recently reported a method of precipitation of cystin by acetic acid in the presence of acetone, which in his hands gave very satisfactory results.

We have estimated the cystin by this method slightly modified, and although there are discrepancies in the results of some of the duplicate determinations, we have decided to publish them, and an account of the limits of accuracy and technic employed will be given.

The total nitrogen determinations have all been made in duplicate by the Kjeldahl method. Urea determinations have also in every case been made in duplicate, using the method of Folin.

Ammonia, creatin and creatinin have been estimated by the methods of Folin. Uric acid was estimated by the Folin-Shaffer method.

Total sulfur, inorganic, ethereal and neutral sulfur have been determined by Folin's methods.

Diamines have been sought by the method of benzoylation,[2] and by the method of precipitation with phenyl-isocyanate.[3]

Other amino-acids have been sought in a mixture of urines from which cystin had been removed by Gaskell's method. The method employed will be described under the caption "Other Amino-acids" later.

1. *Relation of Cystin Excretion to the Amount of Protein Food Ingested.*

On account of the long history of gastric indigestion and the fact that the patient was obliged to continue at work during the course of the investigation, we were disinclined to disturb his diet more than was absolutely necessary to secure the information desired.

He was already on a diet which was practically constant, consisting of milk, eggs and biscuit, and an inspection of the table shows at once the very slight amount of variation in the daily nitrogen excretion from No. 855 to No. 858 inclusive. During this period he was taking the diet to which he had been accustomed for many months.

[1] Gaskell: *Journ. of Physiol.*. xxxvi, p. 142, 1907.

[2] Baumann and Udransky: *Zeitschr. f. physiol. Chem.*, xiii, p. 562.

[3] Herzog: *Zeitschr. f. physiol. Chem.*, xxxiv, p. 524, 1901. Loewy and Neuberg: *Ibid.*, xliii, p. 352, 1905.

The *average* total nitrogen output *per diem* during this period was 9.62 grams.

Beginning with No. 859 and continuing for four successive days, he took five eggs in addition to his customary diet. Besides the eggs, we gave him on the first two of these days, 25 grams of "Somatose" in solution, and on the latter two days 50 grams of the same substance in solution. This substance, which contains 12.8 per cent of nitrogen, according to determination made on a sample of the specimen used, was administered for the purpose of largely increasing the nitrogen intake, without unduly increasing the bulk of the ingesta.

He not only bore this addition to the diet without detriment, but retained a considerable part of the nitrogen.

The *average* total nitrogen output on these four days was 11.28 grams, which shows that he was actually catabolizing a larger amount of protein.

Referring again to the table, we find that on the first four days, i. e., while on his usual diet, the average daily output of neutral sulfur was 0.322 gram, while on the four succeeding days the average excretion *per diem* was 0.375 gram.

If we may be permitted to consider that the increase of neutral sulfur is due to increase in the amount of cystin excreted, then this difference of 0.053 gram of neutral sulfur *per diem* during the four days of increased protein catabolism, will represent an increase in the cystin output of nearly 0.2 gram *per diem*. When we enter upon the discussion of the actual determinations of cystin, we shall show that after making due allowance for inaccuracies in the analytical results the weight of evidence is strongly in favor of the assumption that this increase is mainly due to increase in the cystin output.

This result is in consonance with the results obtained by Alsberg and Folin in a similar experiment, where the difference in the amount of protein in the two diets was much greater, and also agrees with the results of Wolf and Shaffer.[1]

Thiele was led to the opposite conclusion as a result of his work, but drew his conclusions from the results of change in diet on single days only. The fallacy of attempting to draw such a conclusion from experiments with diets of varying composition dur-

[1] *Loc. cit.*

ing periods of twenty-four hours only may be easily inferred from an inspection of the daily neutral sulfur output during the four days of increased protein feeding in this case. It will be noted that while the neutral sulfur rose to 0.398 on the first day of the experiment, it was only 0.353 on the second day, rising again to 0.370 on the third day and reaching 0.421 only on the last day of the series.

If we compare the relation between neutral sulfur and total sulfur in the two series, we find that during the four days of ordinary diet the neutral sulfur formed 42 per cent of the total sulfur, taking the averages.

During the four days of high protein diet, the average amount of neutral sulfur was only 36.7 per cent of the average total sulfur.

This again corresponds with the results of Alsberg and Folin and with those of Wolf and Shaffer.

2. *The Ability of the Patient to Oxidize Cystin Administered as such per Os.*

Regarding this point we have performed but one experiment. This consisted in the administration on December 1, 1908, of 5 grams of cystin, prepared from human hair. The purity of this cystin had been previously determined by determinations of nitrogen and sulfur and by its microscopic appearance.

This cystin was given in three equal portions, the first at 7 a.m., the second at noon, and the third at 5 p.m. In this case as on all the other days the collection of the specimen was begun at 7 a.m. on the day of the experiment and continued until 7 a.m. of the following day.

On this day and for the five days preceding, he was taking the usual diet to which he had been accustomed before the investigation was begun: that diet which he took on the four days beginning with No. 855.

	Total S.	Inorganic S.	Neutral S.
Sum of two days preceding administration of cystin	1.845	0.995	0.659
Sum of two days following administration of cystin	2.596	1.762	0.767
Increase following cystin administered by mouth	0.751	0.767	0.108

From the above table it will be seen that the administration of this amount of cystin caused an increase in the total sulfur elimination during the 48 hours immediately succeeding of 40.7 per cent.

If, as in the case of Neuberg and Loewy's patient, the cystin administered were excreted in anything like quantitative amount in the urine, we should expect to find very little difference in the amount of inorganic sulfur during the two 48-hour periods. On the other hand we should expect an enormous increase in the neutral sulfur.

We find that the inorganic sulfur excreted during the 48-hour period subsequent to cystin feeding exceeds by 0.767 gram that excreted during the previous 48 hours, the diet, with the single exception of the cystin, being the same throughout the four days. *That is, we have an increase in the inorganic sulfur of 77 per cent.*

In the case of the neutral sulfur there is an increase following cystin administration of 0.108 gram or 16.3 per cent. Interpreting this increase as cystin, we should infer that a moderate degree of intolerance is indicated, but it is perfectly evident that in the case of this patient, the greater part of the cystin absorbed was oxidized and the sulfur excreted as inorganic sulfate in the same manner as occurs when cystin is fed to normal individuals.

3. The Ability of the Patient to Deamidate Other Amino-acids.

For the purpose of examining the ability of the patient to deamidate other amino-acids, he was given on December 7, 1908, 2 grams of tyrosin, divided, as in the case of the cystin, into three equal portions.

The urine collected on that day gave no reaction with Millon's reagent, nor did that collected on the following day.

To make more certain regarding this point, the urine of the day of experiment was evaporated to dryness *in vacuo*, the residue extracted with alcohol to remove the excess of urea, and the residue from this extraction examined microscopically for tyrosin sheaves with negative result.

A portion of the residue was extracted with water at 100° C. and tested with Millon's reagent with negative results.

We therefore feel justified in concluding that this patient is capable of completely deamidating this amount of tyrosin.

There remains, of course, the possibility that the tyrosin administered was not absorbed. No opportunity was had for an examination of the stools, as the patient maintained his daily routine of business throughout the entire experiment and, while willing to collect the urine, declined to save his stools.

4. The Occurrence of Diamines in the Urine.

Two complete twenty-four-hour specimens were devoted to the examination for diamines. Both the method of benzoylation and the method of precipitation with phenyl-isocyanate were employed with negative results.

5. The Occurrence of Other Amino-acids, such as Leucin and Tyrosin in the Urine.

For the purpose of determining the presence or absence of other non-sulfur-containing amino-acids, in particular leucin and tyrosin, the residues of urine from the determination of cystin by Gaskell's method were saved and mixed together.

The acetone was removed by fractional distillation at 57° C. and the mixture of urines thus freed from acetone was precipitated with basic lead acetate until no more precipitate formed. The precipitate having been filtered off and rejected, the excess of lead in the filtrate was removed by precipitation with hydrogen sulfid, this precipitate filtered off and the clear filtrate evaporated *in vacuo* to a thick syrup. This was allowed to stand several days until crystallization occurred. The mass was extracted several times with cold alcohol for periods of about 48 hours, the residue dissolved with hot water and allowed to recrystallize. The crystals were examined microscopically and a portion tested with Millon's reagent.

No trace of leucin or tyrosin could be identified.

6. Is the Increase in Neutral Sulfur in Cystinuria Due Entirely to Cystin?

To answer this question it is necessary that we have a fairly reliable method for determining cystin and the most convincing method would be one in which the cystin is separated from the urine, purified, and actually weighed as cystin. Such a method has been furnished by the investigations of Gaskell.

Gaskell recommends the filtration of the entire twenty-four-hour specimen and a separate determination of the sedimentary cystin.

As we wished to make determinations of total sulfur and total nitrogen, we did not care to do this, especially as we had at the beginning of the work no experience of this method of determination of cystin.

The method we employed was as follows:

Two hundred and fifty cubic centimeters of urine were rapidly measured off after thoroughly agitating the specimen to distribute the sediment as uniformly as possible. The sedimentary cystin was then thrown down in a large centrifuge, the clear urine decanted off and the sediment dissolved in 25 cc. of 2.5 per cent ammonia. This solution was added to the clear urine, which was then treated with 5 cc. of strong ammonia and 20 cc. of 20 per cent calcium chloride solution to precipitate phosphates and oxalates. The whole was then centrifuged until the bulky precipitate was compressed into a compact mass from which perfectly clear liquid could be poured off.

We have added to the original 250 cc. of urine, 50 cc. of reagents, all accurately measured. If, therefore, we take of this clear liquid 240 cc. or four-fifths the amount of urine and reagents, we have four-fifths of the original 250 cc. of urine, or 200 cc. This familiar procedure is of great advantage in this determination, as the bulky precipitate filters slowly and is difficult to wash.

The amount of calcium chloride to be added was determined by ascertaining the amount required to precipitate completely a measured amount of a mixture of the urines to be examined and taking a moderate excess. We also determined that calcium chloride gives no precipitate in 50 per cent acetone with addition of acetic acid after standing for a week.

Having obtained the clear liquid free from excess of phosphates and oxalates, the procedure of Gaskell was followed, the cystin being precipitated by acetic acid in the presence of an equal volume of acetone, allowing four or five days for completion of the precipitation.

The precipitate was then re-dissolved in 2.5 per cent ammonia and re-precipitated with acetic acid and acetone, twenty-four hours being required for the second precipitation.

Many of these determinations were done in duplicate and, while in some cases the correspondence of results was close, in others there was a considerable discrepancy. Unfortunately the largest discrepancy occurred in No. 868, the specimen of the day of cystin administration.

Three determinations were made of the cystin in this specimen and the result given in the table under No. 868 is the average of

the two in closest correspondence. The error amounts to 5 per cent of the twenty-four-hour amount of cystin, and while this is far from an excellent analytical result, the significance of the figures would not be altered whichever result were chosen. It has consequently seemed proper to record the average with this explanation.

The other duplicates were all in error less than 5 per cent and in nearly all cases less than 2 per cent and in all cases the average is given.

To test the purity of the precipitate weighed as cystin, total nitrogen was determined by the Kjeldahl method in one of the specimens, and calculating from the nitrogen value obtained back to cystin gave a result 2.2 per cent in error.

The purity of the precipitates was further investigated as to possible contamination by inorganic substances. On ignition it was completely burned, leaving no weighable residue.

Microscopically, the precipitate occurred in hexagonal crystals during the first slow precipitation from the urine. The second precipitation, which occurred more rapidly, gave a mixture of hexagonal and needlelike forms.

To obtain information regarding the accuracy of the Gaskell method, we repeated one of Gaskell's experiments. This consisted in mixing together several specimens of cystinuria urine and making duplicate determinations of the cystin after filtering off and rejecting any sedimentary cystin. These duplicates corresponded within less than 0.2 per cent.

To a portion of the same specimen of mixed and filtered cystinuria urines was added 0.1 gram of pure cystin, prepared from human hair.

	Gram of cystin.
There was recovered	0.1274
Average of duplicate determinations	0.0224
Difference (should equal cystin added)	0.1050

This result is 0.39 per cent in excess of what should have been obtained.

Aside from the filtering off of the sedimentary cystin, the method used in these control determinations was the same as

given above, which leads us to believe that the method as outlined by Gaskell is accurate, and that we fell into error in attempting to make the determinations in unfiltered urine. We think, however, that where a centrifuge of sufficient capacity is available, the modification of taking an aliquot part of the liquid after precipitation by calcium chloride results in great saving of time without sacrificing accuracy.

Bearing in mind the limit of accuracy which we have determined for our results in the cystin determinations, we may proceed to inspect the tabulated figures. We see at once that if we accept one gram as the amount of neutral sulfur normally excreted *per diem*, the difference between cystin sulfur and neutral sulfur in nearly every case is in excess of this. Indeed, in more than half the determinations it is found to be more than double the amount of neutral sulfur found in normal urines. This undoubtedly indicates that the neutral sulfur fraction in cystinuria is increased not only by the cystin sulfur, but by the sulfur of other substances, either an increased amount of those which make up the normal neutral sulfur, or possibly by products which, like cystin itself, do not occur in demonstrable quantity in normal urine. Traces of such a substance were shown to be present in a cystinuria urine investigated by Spiegel.[1]

It is interesting, moreover, to note that with the increased output of cystin during the four days of high protein diet, the quantity of other neutral sulfur-containing substances was not relatively increased, but on the other hand was relatively decreased.

The excess of neutral sulfur not represented by cystin was well marked during the period of high protein diet, but we found that the excess of neutral sulfur *per diem* during that period, as compared with the four preceding days, was only 0.053 gram.

The excess of cystin sulfur is found to average 0.072 gram *per diem*. Allowance for a possible error of 2 per cent in the cystin determinations does not alter this figure appreciably. On a high protein diet, then, a greater proportion of the neutral sulfur is excreted as cystin than is the case on a low protein diet.

It will also be profitable to review the effect of high protein

[1] Spiegel: Virchow's *Archiv*, clxvi, p. 364, 1901.

No.	Date. 1908	Volume.	Total nitrogen.	NITROGEN AS						PER CENT OF TOTAL NITROGEN.						SULFUR AS		
				Urea.	Ammonia.	Creatinin.	Creatin.	Uric Acid	Undetermined.	Urea.	Ammonia.	Creatinin.	Creatin.	Uric Acid.	Undetermined.	Total.	Inorganic.	Ethereal.
855	Nov. 11	930	9.35	7.22	0.41	0.54		0.116	1.06	77.22	4.38	5.76		1.24	11.4	0.744	0.391	0.0
856	Nov. 12	980	9.67	7.43	0.39	0.57		0.137	1.15	76.85	4.03	5.87		1.42	11.83	0.757	0.372	0.0
857	Nov. 13	1060	9.83	7.71	0.34	0.54		0.137	1.1	78.4	3.45	5.49		1.39	11.27	0.785	0.420	0.0
858	Nov. 14	1220	9.64	7.26	0.43	0.56		0.183	1.22	75.3	4.43	5.83		1.9	12.61	0.776	0.385	0.0
859	Nov. 18	1200	10.96	8.72	0.35	0.55		0.169	1.17	79.4	3.17	5.04		1.54	10.68	1.066	0.599	0.0
860	Nov. 19	1200	11.6	8.34	0.534	0.62		0.122	1.98	71.8	4.6	5.33		1.05	17.06	1.013	0.605	0.0
861	Nov. 20	1400	11.34	9.48	0.46	0.59		0.131	0.68	83.53	4.07	5.18		1.16	5.99	1.019	0.595	0.0
862	Nov. 21	1100	11.22	8.39	0.43	0.57		0.142	1.69	74.89	3.81	5.09		1.27	15.05	1.095	0.598	0.0
863	Nov. 23	1000	11.1	8.03	0.42	0.65		0.159	1.84	72.32	3.78	5.85		1.43	16.57	0.925	0.561	0.0
864	Nov. 24	870	10.62	8.21	0.58	0.61		0.105	1.11	77.31	5.49	5.78		0.983	10.44	0.873	0.534	0.0
865	Nov. 25	1070	10.06	7.99	0.47	0.56		0.118	0.92	79.4	4.68	5.54		1.17	9.14	0.837	0.491	0.0
866	Nov. 27	890	11.13	8.49	0.41	0.62	0.043	0.0997	1.47	76.03	3.67	5.58	0.38	0.895	13.21	0.977	0.531	0.0
867	Nov. 30	840	11.08	8.28	0.54	0.61		0.099	1.55	74.75	4.85	5.46		0.894	13.98	0.868	0.464	0.1
868	Dec. 1	1210	10.73	8.38	0.49	0.56		0.133	1.17	78.14	4.56	5.24		1.24	10.91	1.683	1.240	0.0
869	Dec. 2	1050	10.08	6.92	0.78	0.58		0.121	1.68	68.54	7.76	5.78		1.2	16.67	0.913	0.522	0.0
873	Dec. 3	1160	10.15	7.36	0.67	0.57		0.145	1.4	72.47	6.63	5.66		1.43	13.79	0.843	0.422	0.0
877	Dec. 4	995	9.69	6.71	0.51	0.55		0.138	1.78	69.23	5.27	5.7		1.42	17.98	0.822	0.448	0.0
878	Dec. 5	1070	11.02	8.42	0.45	0.62		0.149	1.38	76.46	4.04	5.63		1.36	12.52	0.926	0.520	0.0
885	Dec. 7	1480	9.47	7.17	0.35	0.49		0.122	1.34	75.7	3.7	5.16		1.28	14.15	0.756	0.392	0.0
889	Dec. 8	1160	9.8	6.82	0.48	0.54		0.114	1.84	69.6	4.89	5.51		1.17	18.77	0.800	0.407	0.0

SULFUR AS			PER CENT OF TOTAL SULFUR.						
Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.	Cystin.	Cystin sulfur.	Neutral S—Cystin S.	
.391	0.067	0.286	52.6	9.0	38.4	0.433	0.115	0.171	
.372	0.035	0.350	49.2	4.62	46.2	0.314	0.084	0.266	
.420	0.062	0.302	53.5	7.9	38.4	0.545	0.145	0.157	
.385	0.042	0.349	49.6	5.42	45.0	0.447	0.119	0.230	
.599	0.069	0.398	56.2	6.47	37.3	0.631	0.168	0.230	25 grams "Somatose" and 5 eggs added to diet.
.605	0.056	0.353	59.6	5.52	34.9	0.482	0.128	0.225	25 grams "Somatose" and 5 eggs added to diet.
.595	0.044	0.370	58.4	4.32	36.3	0.816	0.217	0.153	50 grams "Somatose" and 5 eggs added to diet.
.598	0.076	0.421	54.6	6.94	38.4	0.882	0.235	0.286	50 grams "Somatose." and 5 eggs added to diet.
.561	0.026	0.338	60.6	2.81	36.5	0.792	0.211	0.127	
.534	0.018	0.321	61.2	2.06	36.8	0.780	0.208	0.113	
.491	0.002	0.344	58.6	0.239	41.2	0.433	0.115	0.229	
.531	0.075	0.371	54.4	7.68	38.0	0.478	0.127	0.244	Ate turkey on preceding day.
.464	0.116	0.288	53.4	13.36	33.2				
.240	0.008	0.435	73.5	0.475	25.8	1.567	0.418	0.017	5 grams cystin administered *per os* in three portions.
.522	0.059	0.332	57.2	6.47	36.4	0.645	0.172	0.160	
.422	0.050	0.371	50.1	5.93	44.0	0.540	0.144	0.227	
.448	0.048	0.326	54.5	5.84	39.7	0.497	0.132	0.194	
.520	0.054	0.352	56.2	5.83	38.0	0.465	0.124	0.228	
.392	0.053	0.311	51.8	7.02	41.2	0.267	0.071	0.240	2 grams tyrosin administered *per os* in three portions.
.407	0.067	0.326	50.8	8.37	40.7	0.435	0.116	0.210	

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diet on the cystin excretion, taking the actual determinations of cystin instead of the neutral sulfur as a criterion. The average daily cystin excretion on the usual diet was 0.435 gram. During the four days of high protein diet the average daily excretion of cystin was 0.703 gram, an increase of 0.268 gram of cystin *per diem* on the high protein diet. This amply bears out the conclusions reached from consideration of the sulfur partition and, taken with the results of Alsberg and Folin and also those of Wolf and Shaffer already mentioned, seems to establish the fact that increased protein catabolism in cystinuria is attended with increase in the cystin excretion.

THE INDIRECT COLORIMETRIC DETERMINATION OF PHOSPHORUS WITH URANIUM ACETATE AND POTASSIUM FERROCYANIDE.

BY ROBERT B. GIBSON AND CLARENCE ESTES.

(*From the Laboratory of Physiological Chemistry, Department of Physiology, University of Missouri.*)

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The application of colorimetric principles to the determination of phosphorus is especially desirable in view of the long drawn out and laborious gravimetric method now customarily employed. The accuracy of colorimetric methods, the ease with which most colorimetric procedures are carried on and their employment where only small amounts of material are available for analysis would make a reliable colorimetric reaction for phosphorus of the greatest value. Titration with uranium acetate is extensively used for the urinary phosphates and sufficient accuracy is obtained for ordinary metabolism investigations. Titration of the ammonium molybdate precipitate after adding an excess of $\frac{N}{10}$ sodium hydroxide or potassium hydroxide (Neumann) is stated by Gregersen[1] to be accurate to 1 milligram of phosphorus when certain technical modifications of the original method are followed.

The molybdate reaction for color comparison in the determination of phosphorus has been employed by Schreiner,[2] by Woodman and Cayvan[3] and by Veitch.[4] The intensity of the color developed is influenced to some extent by temperature (Woodman and Cayvan). Silicates give a similar reaction, and their presence in the ordinary laboratory reagents introduces error in the determinations. Veitch has shown that ammonium salts and certain chlorides influence the color of the phosphomolybdate solutions. One of us (Estes)[5] has recently reported experi-

[1] Gregersen: *Zeitschr. f. physiol. Chem.*, liii, p. 453, 1907.
[2] Schreiner: Bull. No. 31, Bureau of Soils, U. S. Dept. Agr., 1906.
[3] Woodman and Cayvan: *Journ. Amer. Chem. Soc.*, xxiii, p. 26, 1901.
[4] Veitch: *Ibid.*, xxv, p. 169, 1903.
[5] Estes: *Journ. Amer. Chem. Soc.*, xxxi, p. 247, 1909.

ments showing the percentage error introduced by foreign salts in the colorimetric determination of phosphates. Sodium sulphate, acid potassium sulphate, magnesium sulphate, sodium chloride and an excess of nitric acid all greatly reduce the intensity of the color; when these substances are present in high concentration, only a trace of color appears, and the errors found are not directly proportional to the weight of the salt added. The nitrates of calcium and magnesium exhibit a different effect; the color is diminished up to a concentration of 14 per cent and 8 per cent of the two salts respectively, and is increased in stronger solutions so that a nearly normal comparison can be obtained. The oxidation of organic matter with magnesium nitrate[1] gives relatively small error in the phosphomolybdic color comparison.

In the present paper we report the results of some experiments to find a procedure[2] for the colorimetric determination of phosphorus that would be more generally applicable than the limited phosphomolybdate method. The addition of an excess of uranium acetate solution to an aliquot of the diluted fusion or digestion and the determination of the uncombined uranium colorimetrically after decanting or filtering from the precipitated uranium phosphate seemed most promising. While it is possible that the idea may have been subjected to experimental test before, we know of no determinations reported in which this procedure has been employed.

Some earlier determinations on stock solutions of disodium phosphate, using the ordinary standard uranium acetate (diluted 1:100) and the special sodium acetate acetic acid solution described below, indicated that the method might be more generally applicable. Practically absolute accuracy was obtained with determinations on the phosphate solution against which the uranium acetate was standardized by titration. For example, three determinations are given:

Grams P_2O_5 taken.	Grams P_2O_5 found.	Uranium sol. used.	Ppt'd mix. made up to
0.0006795	0.0006795	20 cc. 1:100	40 cc.
0.0005436	0.0005436	25 cc. 1:100	50 cc.
0.0054360	0.0054360	40 cc. 1:100	80 cc.

[1] Wiley: *Agricultural Analysis*, i, p. 587, 1906.

[2] A preliminary report was made earlier. Cf. *Proc. Amer. Soc. Biol. Chem.*, this *Journal*, vi, no. 2, p. xxv, 1909.

Of the accessory solution 5 cc. were used in the precipitation; 20 cc. of the filtrate, acidified with two drops of acetic acid, and 1 cc. of 10 per cent. potassium ferrocyanide were made up to 100 cc. and contrasted with a "blank" precipitation.

Observations with known amounts of phosphate added to blank alkali fusions or acid digestions indicated that the conditions under which such determinations could be made would have to be controlled to some degree; especially so as regards the conditions affecting the development of the uranium ferrocyanide color reaction. The following procedure was finally adopted as the most satisfactory.

Solutions. (1) A standard uranium acetate solution. The usual solution employed in the titration of phosphates, containing 35.461 grams of uranium acetate per liter, and carefully standardized by titration (with ferrocyanide as the indicator) against a disodium phosphate solution equivalent to 0.005 gram of P_2O_5 per cc. Of the uranium acetate solution, 20 cc. are diluted to 1000 cc. for the colorimetric determinations.

(2) A special sodium acetate acetic acid solution. This is prepared by dissolving 20 grams of sodium acetate in distilled water, adding 100 cc. of 30 per cent acetic acid and making up to 1000 cc. It is the ordinary accessory solution containing a fifth the customary amount of sodium acetate.

Fusions. Alkali fusions may be made in the usual manner with 25 grams or less of the fusion mixture (4 parts sodium hydrate and 1 part potassium nitrate) according to the quantity of material to be oxidized. These are more difficult to burn and are not so satisfactory for the determination of phosphorus colorimetrically as the sulphuric acid digestions because of the possible excess of nitrates which intensify the color reaction. We have not employed the sodium peroxide oxidation now so extensively used; there is no reason why this procedure should not be preferred to the ordinary alkali fusion for the colorimetric determinations. The fusions should be neutralized with *sulphuric acid*, the acid added in excess, the mixture diluted somewhat and boiled a few minutes to drive off the nitric oxide. When small aliquots (10 cc. of a fusion diluted to 250 cc.) can be used, the fusions can be neutralized with hydrochloric or nitric acid; in case

it is desirable to employ larger aliquots,[1] equivalent amounts of sodium chloride or sodium nitrate should be added in making up the colorimetric blank with which the determination is to be contrasted. Quantitative dilutions of the alkali fusions are conveniently made to 100 or 250 cc.

Sulphuric acid and nitrate digestions are made as directed in the *Official and Provisional Methods of Analyses* of the Association of Official Agricultural Chemists. More rapid oxidation may be attained by heating the sample with 20 cc. of concentrated sulphuric acid in a Kjeldahl flask until the water is driven off and the organic matter charred, allowing the digestion to cool, adding 5 cc. of fuming nitric acid and heating moderately until the excess of nitric acid is distilled off.[2] If oxidation is not then complete, a crystal or two of ammonium nitrate may be thrown in and the heating continued. The digestions should be diluted and boiled for a few minutes. The acid digestions should be quantitatively diluted to 100 or 250 cc. but without neutralization. There seems no reason why aliquots of Kjeldahl-Gunning nitrogen determinations should not be employed.

Precipitation with Uranium Acetate. Aliquots, preferably one-twenty-fifth to one-tenth according to the P content of the diluted fusions are pipetted into Florence flasks, the necks washed down with a little distilled water, a small piece of litmus paper thrown in and ammonia solution (sp. gr., 0.90) added in excess of neutrality. The flasks are then boiled over a low flame until the litmus paper again turns red and the solution is concentrated to 10-15 cc. Five cubic centimeters of the special acetate solution are added and exactly 50 cc. of the dilute uranium acetate solution run in slowly from a burette. The mixture is transferred and made up to 100 cc. in an accurately standardized volumetric flask; after mixing, the determination is allowed to stand over night for complete precipitation. A solution containing 50 cc. of the uranium acetate and 5 cc. of the accessory mixture is similarly made up to 100 cc. for the known contrast.

Colorimetric Comparisons. Exactly 10 cc. of the decanted supernatant fluid or of the filtrate (obtained perfectly clear by

[1] When sulphur is to be determined on the same fusion and neutralization with sulphuric acid is impractical.

[2] This end point is not reached when the colored fumes cease to evolve.

pouring on the paper several times if necessary) from the uranium phosphate precipitation are pipetted into an accurately standardized 100 cc. volumetric flask and 1.5 cc. of 10 per cent potassium ferrocyanide solution added. The neck of the flask is washed down with a little water. The red-brown color is completely developed, usually in the course of a couple of minutes; where large aliquots of the fusion have been employed, there may be sulphate present in sufficient amount to delay the complete development of the color reaction for 10 or 15 minutes. The flask is filled up to the 100 cc. mark with distilled water and thoroughly mixed. It is contrasted colorimetrically with 10 cc. of the blank "precipitation" similarly treated. The color is permanent for several hours. The contrasts are very sharp and fair comparisons can be made even by artificial illumination.

Calculation. The number of cc. of the uranium acetate solution in the filtrate from the uranium phosphate precipitation is given by the following colorimetric proportion:

$$\text{Unknown: Known: : 50 cc: } x \text{ cc.}$$

Hence $50-x$ is the number of cubic centimeters of the uranium acetate solution, the uranium of which has been combined with the phosphate of the aliquot taken. The exact equivalent of 1 cc. of the uranium acetate solution as P_2O_5, PO_4 or P being known, the total amount or per cent in the sample is readily obtained.

Analyses of Casein. Sulphuric acid digestions of 2.5000 grams of a commercial preparation were made in duplicate, and the dilutions made to 250 cc. Two hundred cubic centimeters of each were taken for gravimetric determinations in duplicate for each acid digestion. A colorimetric determination was made on a 10 cc. aliquot of each fusion, and in neither instance was there any duplication in the course of the colorimetric analyses.

	Colorimetric.		Gravimetric.	
	P found grams.	P found per cent.	P found grams.	P found per cent.
1	0.0264	1.055	0.0261	1.044
2	0.0269	1.075	0.0268	1.073

Analyses were also made on acid digestions of the same casein sample (using 1.7093–2.0000 grams for the individual oxidations). A single colorimetric determination was done on a 10 cc. aliquot

of each digestion diluted to 250 cc. without further duplication of any sort. Gravimetric determinations were not made, the results of the analyses in the table above being taken as sufficient control. Four analyses gave 1.118, 1.107, 1.058 and 1.137 percents respectively; a fifth, to which 0.4662 gram of hemoglobin had been added, contained 1.092 per cent of phosphorus after correcting for that of the hemoglobin which was determined gravimetrically. Three alkali fusions[1] of the same sample gave 1.122, 1.055 and 1.092 per cents of phosphorus.

Salmon Tissue. Analyses were made on some samples furnished us by Prof. C. W. Greene of this department. The entire sample in each instance was oxidized by acid digestion. The amounts of phosphorus, however, in three instances were too small for satisfactory duplicate gravimetric analysis, and for two of the samples the duplicate precipitates of ammonium magnesium phosphate were pooled; the other determination necessitated the weighing of less than 7 mgm. of magnesium pyrophosphate. It is possibly of course merely a coincidence that this analysis (no. 4) was the one which should show the greatest difference from the colorimetric results. The figures are given for the total phosphorus in the sample. In each instance the digestions were made up to 250 cc., 100 cc. aliquots being taken in duplicate for gravimetric work and 10 and 20 cc. aliquots for colorimetric phosphorus.

	Colorimetric. Grams P.		Gravimetric. Grams P.	
Tissue.	10 cc. al.	20 cc. al.	1	2
1. Skin	0.0122	0.0126	0.0127	0.0129
2. Liver	0.0068	0.0072	0.0074	0.0074
3. Muscle	0.0026	0.0023	0.0030	
4. Testes	0.0038	0.0037	0.0047	0.0049
5. Muscle	0.0027	0.0025	0.0028	
6. Gut	0.0075	0.0071	0.0075	0.0077

Wheat. Duplicate acid digestions were made on 1 gram amounts of wheat which had previously been analyzed for phosphorus. The digestions were diluted to 100 cc. and a 10 cc. aliquot of each taken for a colorimetric determination. By the earlier gravimetric analyses, the sample contained 0.42 per cent

[1] These were neutralized with nitric acid.

of phosphorus; the single colorimetric determination on each of the two digestions gave 0.394 and 0.404 per cent.

The influence of salts and of the acidity in the molybdate colorimetric method, as stated in the earlier portion of the paper, leads to considerable variation in the analytical results obtained. In the dilutions and aliquots that we have employed in the above analyses by the uranium acetate colorimetric method no interference of this kind is encountered. Nitric acid and nitrates, and to slightly less degree chlorides, intensify the color in the uranium-ferrocyanide reaction to a noticeable extent, a maximum and constant point being quickly attained at comparatively low concentrations of the salts. Sulphates delay the development of the color slightly and in high concentrations have an inhibiting action. The influence of nitrates, nitrites, chlorides and sulphates on the color reaction with blank fusions is shown in the accompanying table.[1]

Excess of acetic acid or of sodium acetate (added at the time of precipitation with uranium) inhibits the uranium-ferrocyanide color reaction, for which however an acid reaction is necessary. Mineral acids would lead to the formation of the Prussian blue reaction in the presence of iron in the sample. By the method outlined, the acidity is exactly the same in both the control contrast and the precipitated aliquot; the concentration of H ions is sufficiently reduced by the acetate added to prevent the interference of iron in the determination.

The results which we have obtained by this indirect colorimetric method for phosphorus have given us confidence in its accuracy and general applicability. The ease and rapidity with which the analyses can be made have been especially brought home to us in checking up the colorimetric with gravimetric determinations. With exactly standardized volumetric apparatus and one of the better colorimeters now on the market, even more accurate results than those we have reported seem possible.

In the precipitation of phosphates with uranium in the presence of ammonium salts, the precipitate may contain ammonia as indicated by the formula: $UO_2NH_4PO_4$. The ratio of uranium to phosphorus is of course

[1] The reagents were taken in the quantities indicated without actually fusing the mixtures.

The Influence of Nitrates, Chlorides and Sulphates on the Uranium-Ferrocyanide Reaction.

BLANK OXIDATION WITH	NEUTRALIZATION WITH	ALIQUOTS OF OXIDATIONS DILUTED TO		COLOR INCREASE*	REMARKS.
		100 cc.	250 cc.		
		cc.	*cc.*		
20 gms. NaOH and 5 gms. KNO_3	Conc. HNO_3	4	10	none.	
		10	25	100:94	
		20	50	:93	
		40	100	:93	
		10	25	:97	0.5 gm. $(NH_4)_2 SO_4$ added in developing the ferrocyanide reaction.
		40	100	:98	1.0 gm. $(NH_4)_2SO_4$ added in developing the ferrocyanide reaction
	Conc. HCl	4	10	none	
		10	25	100 : 96	
		20	50	: 96	
		40	100	: 96	
	H_2SO_4	4	10	none	
		10	25	"	
		20	50	"	Color reaction slightly delayed in reaching maximum intensity.
		40	100	"	Color maximum after about 15 min. contrasted after 30 min.
40 cc. H_2SO_4 sp. gr. 1. 84	NH_4OH	10	25	none	
		20	50	"	
		25	62	"	Color maximum after about 15 min.
		40	100	—	Color reaction practically inhibited.
20 cc. H_2SO_4† 5 cc. HNO_3	NH_4OH	4	10	none	
		10	25	100 : 95	
		20	50	: 95	
		40	100	: 94	Color reaction noticeably delayed in reaching maximum intensity.
20 gms. NaOH and 5 gms. KNO_2	H_2SO_4	10	25	100:108	

* The readings in millimeters are inverse to intensity of color. The control was read at 70 mm. and the readings calculated for 100 mm.

† Heated until colored fumes ceased to evolve.

not affected in the reaction. It seemed possible that we had here a means which might be applied to advantage to combine the determinations of phosphates and of ammonia in the urine. Accordingly, the uranium phosphate precipitates obtained in the ordinary phosphate titrations were filtered and washed, precipitates and papers transferred to flasks, an excess of NaOH solution added and the mixture distilled into $\frac{N}{10}$ acid. Only about one-third of the ammonia present (Folin-Shaffer) was obtained. Larger quantities of ammonia were obtained when phosphate and equivalent uranium acetate solutions were added to the urine.[12] The procedure now becomes more complicated; but even if reliable, it would not be so satisfactory as the Folin-Schaffer method ordinarily used.

[12] These analyses were made by Mr. C. H. Hecker.

NOTES ON THE EFFECT OF SHAKING UPON THE ACTIVITY OF PTYALIN.

By MARIE M. HARLOW and PERCY G. STILES.

(*From the Biological Department of Simmons College.*)

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The observations of Shaklee and Meltzer[1] on the effect of shaking upon pepsin suggest much related work to be done with other enzymes. These authors found that pepsin could be totally destroyed by vigorous and prolonged shaking and that even the movements of an animal might suffice to weaken in a marked degree a solution of the enzyme enclosed in a vial and introduced into the stomach.

The loss of activity of digestive solutions which have undergone shaking is not due to exposure to air. It may conceivably be due to molecular changes either of a disintegrative character or in the nature of a clumping into inert aggregates. It is likely to be due, at least in part, to the effect of contact with surfaces. So far as it is of this type it is analogous to the removal of enzymes from solutions by precipitates and by filtration.[2] There is in any case the possibility that under some circumstances the lost power of the enzyme may be regained.

Diastatic enzymes are among the most convenient for such studies because the time required for the digestion of a starch paste to reach the "achromic point" (i. e., to pass beyond the erythrodextrin stage) is a measure of activity easily obtained.

For shaking we used a wooden rack, made to hold six bottles, and suspended by pieces of tape. To the rack was attached a horizontal connecting-rod from a crank turned by a small water-motor. The crank-pin described a three-inch circle about 300 times a minute.

[1] Proc. Amer. Physiol. Soc., *Amer. Journ. of Physiol.*, xxiii, p. xxix, 1909.

[2] Levy: Sixth Annual Report, Michigan Academy of Science, 1906, p. 155.

Our first attempts to affect undiluted saliva by shaking for an hour or two gave negative results. We then diluted the saliva in the ratio of 1:10. Shaking such a solution in a plain bottle does not cut down its hydrolytic power very rapidly. We anticipated that more surface exposure would have an influence and for the purpose we introduced some glass beads. The reduction of digestive power became marked. Continuing our experiments we found that the paralyzing effect upon the enzyme is most considerable in the first half-hour of shaking and that it proceeds thereafter at a diminishing rate. We have never completely destroyed the activity of saliva by shaking with one set of beads. We have often reduced its strength to one-half or one-third the original by this method.

The behavior of the enzyme when treated in this way gave ground for believing that adsorption upon the beads must be an important factor in the process. The lessening effect of shaking for successive periods seemed to indicate a saturation of the glass surface. Accordingly we compared the reduction of efficiency secured by continued shaking with one set of beads with that produced when the solution was poured from one set to another at intervals of a half-hour or less. This repeated introduction of clean glass surfaces greatly increased the paralyzing effect upon the ptyalin. By the use of fresh beads we could keep on cutting down the digestive power of the liquid by a noticeable percentage at each trial. But within the range of our observations some activity always remained.

The importance of increasing the surface was emphasized in one experiment in a way which we had not anticipated. We had previously noted that beads once used to weaken the action of saliva must be very thoroughly cleaned or they will be comparatively ineffective in a fresh experiment. Prolonged rinsing with cold water will not answer the purpose. One set of beads which had been used to neutralize the enzyme were prepared for a later trial by boiling with caustic soda and then with successive waters. These beads when shaken produced a turbid solution and showed a very exceptional power to reduce the activity of saliva. It appeared that the treatment with alkali had rendered the surface of the beads friable and that when shaken they parted with countless scales, giving a greatly increased surface exposed to the

solution. The microscope confirmed the inference, showing the sediment in the bottle to consist of angular particles not altered by boiling.

Another set of beads, deprived of their virtue by use in a previous experiment, we cleaned for repeated use by heating them to redness. Beads prepared in this way showed *less* than an average effect on saliva. It seemed probable that in this case the surface was smoothed and polished by the flame, a result opposite to that secured with the caustic soda. We found, as there was now reason to expect, that wisps of glass wool are more effective, weight for weight, than beads in the shaken samples.

The enzyme in saliva is of course accompanied by other organic substances, such as mucin. We thought that such bodies might have a part in the interaction of the enzyme with surfaces. So we made a few experiments with "Taka-diastase," an amylolytic preparation from a vegetable source (*Aspergillus oryzæ*). The accompanying bodies in Taka-diastase must be quite different from those in human saliva. We found that a one per cent solution of the dry Taka-diastase of commerce has about the strength of a 1:10 dilution of saliva. Shaking with clean beads or glass-wool has a nearly equal effect on the two digestive mixtures. There is therefore no reason to attribute a specific action to the mucin of the saliva.

It is an important practical question whether an enzyme can be as readily destroyed (or paralyzed) in the presence of its normal substrate as when shaken alone. Shaklee and Meltzer's observation that there is motion enough in the stomach of an active dog to impair the powers of pepsin carried about there *in vitro* is very curious. Is there a constant waste of enzymes in the alimentary tract or is the glass surface the destructive factor? Certainly the glass has much to do with the result but we must not forget the isolation of the enzyme from food, which is another unnatural condition. One may imagine that the same agitation which tends to fix the enzyme in a useless film upon glass may help it to engage in relations with its substrate that may promote digestion. After the hydrolysis the active enzyme may reappear. We have shaken saliva and starch together while a similar mixture was digesting unshaken, and have found that the two samples reach the achromic point at about the same time.

A certain protection of the enzyme by its substrate seems probable but is not clearly demonstrated.

Finally, while we are convinced that the removal of the enzyme by contact with surfaces has been the chief factor in our experiments, we have seen some reason to believe in a secondary influence of the shaking, either an agglomeration or a disintegration of molecules it may be. For instance, when we take a volume of saliva which has been greatly weakened by shaking with beads and, dividing it into two parts, leave one portion upon the beads and the other in a plain bottle, we find after an hour or more that the saliva upon the beads continues slowly to grow weaker while the other regains a fraction of its lost strength. The weakening of the sample upon the beads may be due to continued adsorption; the improvement of the other must be due to a re-formation of active molecules, not masked in this case by the surface effect.

THE ESTIMATION OF TOTAL SULPHUR IN URINE.

By STANLEY R. BENEDICT.

(*From the Bowne Hall Laboratory of Physiological Chemistry, Syracuse University.*)

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The estimation of the total sulphur content of urine is an analytical problem presenting some peculiar features. The compounds existing in urine which carry unoxidized sulphur are so stable as to require very vigorous oxidizing agents to convert the sulphur into sulphuric acid, yet the subsequent precipitation of the sulphate must take place in the complete absence of oxidizing agents, as well as of numerous special salts which modify the precipitate. Another requirement in the process is that the oxidation takes place in the presence of some substance capable of forming a stable combination with the newly oxidized sulphur. In the older attempts to fulfill these conditions the urine was burned with an alkali in the presence of an oxidizing agent (nitrate), the latter being subsequently destroyed by repeated evaporation with hydrochloric acid. Folin[1] has called into question the accuracy of the older fusion methods and has proposed a substitute for these which is the method probably most commonly employed at the present time. Like the preceding method of Modrakowski,[2] Folin's process depends upon fusion of the urinary residue with sodium peroxide, and the subsequent destruction of this oxidizing agent through treatment with boiling water. The results obtainable by this method appear to be highly satisfactory, but the technique involved is so laborious and difficult as to make the use of the method, especially where numerous analyses are to be made, almost prohibitive.

Schulz[3] has recently proposed a rapid method for the determination of total sulphur in urine which is based upon oxida-

[1] Folin: This *Journal*, i, p. 131, 1906.

[2] Modrakowski: *Zeitschrift für physiologische Chemie*, xxxviii, p. 562, 1903.

[3] Schulz: *Archiv für die gesamte Physiologie*, cxxi, p. 114, 1908,

tion of the urine with fuming nitric acid. Frequent attempts of the writer and of various analysts under his direction, to make use of Schulz's method have served to demonstrate that this method is totally unreliable and of no practical value. Inasmuch as two criticisms of the method have already appeared in print,[1] it is unnecessary to more than mention here that the writer's results fully confirmed those of Osterberg and Wolf and especially of Konshegg. Konshegg assumes that the low results obtained by Schulz's method are due to a lack of sufficient metallic ions in the urine to act as a base for the newly oxidized sulphur, and he therefore suggests the addition of a large quantity of potassium nitrate, prior to the evaporation with nitric acid. There appears to be no good ground for the assumption at the basis of Konshegg's modification of Schulz's method, for a calculation would show that urine must ordinarily contain at least a hundred times as much sodium as would be required to combine with its neutral sulphur content, calculated as sulphuric acid. In spite of this fact Konshegg advises the addition of a quantity of potassium (as nitrate) many hundred times as great as would be required to combine with the neutral sulphur content of the urine, and further, provides for the precipitation of the barium sulphate in presence of this great excess of nitrate. Since the days of Liebig and Fresenius it has been a matter of common knowledge that sulphate precipitations cannot be carried out successfully in the presence of nitrates or nitric acid. The methods of Schulz and of Konshegg therefore condemn themselves and would be worthy of little discussion, were it not that certain investigators[2] have recently offered results by Konshegg's method as a basis for a general criticism of all previously existing total sulphur methods for urine, laying particuar stress against the method of Folin. The fact that Folin's method gives results differing from those obtained by Konshegg's procedure would, as a matter of fact, appear to be only to the credit of the former process, since Konshegg's method is theoretically incorrect. In attempting to account for the lower results obtained by Folin's method as

[1] Osterberg and Wolf: *Biochemische Zeitschrift*, ix, p. 307, 1908. Konshegg: *Archiv für die gesamte Physiologie*, cxxiii, p. 274, 1908.

[2] Gill and Grindley: *Journal of the American Chemical Society*, xxxi, p. 52, 1909.

compared with that of Konshegg, Gill and Grindley cite experiments in which they found sulphur in the distillates after evaporation as in Folin's process. Folin[1] has replied to this criticism by citing results reported by him which showed that in the case of pure cystin, no loss of sulphur occurs by his process. It is plain that results with cystin yield little evidence that no sulphur compound is volatilized from the urine. According to the description by Gill and Grindley, these investigators carried out the Folin evaporation in a side-necked distillation flask, the outlet tube being led directly into a receiver containing fuming nitric acid. Employing such technique, I have obtained precipitates in the distillates upon addition of barium chloride, either before or after evaporation of the solution with fuming nitric acid and potassium nitrate, both with Folin's procedure and with Konshegg's. It appeared, therefore, to be of interest to repeat these experiments of Gill and Grindley, with the modification of introducing a Kjeldahl bulb between flask and receiver, in order to prevent any possibility of mechanical transfer. In such cases the distillates, after evaporation of the nitric acid (with the addition of potassium nitrate), showed no turbidity upon standing three hours after addition of barium chloride. From these results, verified by a large number of experiments, it will be recognized that the sulphur found by Gill and Grindley in the distillates from Folin's process was obtained by mechanical spattering, and that no loss of sulphur occurs during the evaporations called for in our common total sulphur methods. The adherent of any method which provides for the precipitation of sulphate in the presence of a nitrate must assume the burden of the proof that his results are the correct ones.

In view of the very questionable value of the nitric acid processes for total sulphur estimation, and on account of the difficulties attending the fusion processes, a new method, based upon a different line of procedure, is much to be desired. The following method had its basis in the endeavor to find a substance which, while a vigorous oxidizing agent at certain temperatures, should at the same time be decomposing into a neutral, non-oxidizing compound, which would combine with the oxidized

[1] Folin: *Journal of the American Chemical Society*, xxxi, p. 284, 1909.

sulphur to form a stable combination. Such a substance has been found in copper nitrate. Upon heating this salt it decomposes quantitatively into two vigorous oxidizing agents, nitrogen dioxide and cupric oxide. The former of these compounds, being gaseous, is readily completely removed, while the latter dissolves easily in hydrochloric acid to a solution which exhibits no oxidizing action, nor in any way affects the precipitation of barium sulphate. The copper oxide also furnishes a base for the oxidized sulphur, forming with it a compound which is stable at any temperature of the Bunsen flame. Theoretically, therefore, copper nitrate should afford a highly satisfactory oxidizing agent for the urine as regards total sulphur estimation, and practically it seems to fulfill all necessary conditions. Evaporation of the urine with copper nitrate alone over a free flame is followed by an oxidation which starts with some suddenness and leaves a fluffy residue which tends to fly about very easily. The addition, however, of a small amount of sodium or potassium chlorate overcomes this disadvantage. The oxidation then begins at a slightly lower temperature, proceeds in an orderly fashion, and leaves a residue which shows no tendency to leave the container. Following is the technique and procedure.

REAGENT

Crystallized Copper Nitrate[1]	200.00 grams
Sodium or Potassium Chlorate[2]	50.00 "
Distilled water to	1000.00 cc.

Ten cubic centimeters of urine are measured into a *small* (7 to 8 cm.) porcelain evaporating dish and 5 cc.[3] of the reagent added. The contents of the dish are evaporated over a free flame which is regulated to keep the solution just below the boiling point, so that there can be no loss through spattering. When dryness is reached the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the Bunsen burner and the contents of the

[1] The copper nitrate should be examined for the presence of sulphate, and where found, a blank determination should be made.

[2] Repeated determinations have shown that the results are the same whether the sodium or potassium salt be employed.

[3] If the urine is concentrated, the quantity of reagent should be slightly increased.

dish thus *heated to redness for ten minutes after the black residue* (which first fuses) *has become dry*. This heating is to decompose the last traces of nitrate (and chlorate). The flame is then removed and the dish allowed to cool more or less completely. Ten to twenty cubic centimeters of dilute (1:4) hydrochloric acid are then added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely two minutes. With the aid of a stirring rod the solution is washed into[1] a small Erlenmeyer flask, diluted with cold, distilled water to 100 to 150 cc., 10 cc. of 10 per cent barium chloride solution added drop by drop and the solution allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighed Gooch. A dozen determinations may be made ready for precipitation by this process in considerably under one hour.

In discussing the adequacy of the above method we must first inquire as to what shall constitute a criterion of a total sulphur method for the urine. Were we well acquainted with the neutral sulphur compounds of the urine, it would only be necessary to prove that the method could yield theoretical results with these compounds. Since however, there are such numbers of sulphur-bearing substances in the urine which cannot be obtained in the pure form, we must, for the present at least, be content with a less absolute critique. Folin[2] has said that a total sulphur method must as a first requisite yield six duplicate determinations upon the same sample of urine with a variation of not over 1 per cent of the total sulphur present. To the present writer it appears just as well to require that the duplicates must agree within the limits of error for ordinary gravimetric determinations—say 0.4 mgm., independent of the quantity of sulphur present. A second requisite would be that the total sulphur

[1] Sometimes the porcelain glaze of the dish becomes cracked during the heating, and it is then safer to filter the solution through a small folded filter into the flask, followed by a little wash water. The same filter may be used for any number of simultaneous determinations. Observing the precaution of filtering, the same dishes may be used daily for several weeks without any difficulty.

[2] Folin: This *Journal*, i, p. 156, 1908.

found must be appreciably greater than the sum of inorganic sulphur and ethereal sulphur. Thirdly, no detectable loss of sulphur during the manipulation involved in the process can occur. Fourthly, there must be nothing in the process or in the salts present, which can be shown to influence the precipitation of pure sulphate solutions.

The copper nitrate method above suggested meets these standards satisfactorily. This statement is based upon results of several hundred determinations carried out in exact accord with the above directions. A few examples of the results will be cited. The following are seven duplicate determinations made upon one sample of urine.

No. of Determination	Weight of $BaSO_4$. *Gram.*
1	0.0600
2	0.0602
3	0.0598
4	0.0604
5	0.0601
6	0.0599
7	0.0600

There is no doubt that where carried out with reasonable care, the method yields satisfactory duplicates. As regards the relationship of total sulphur, as obtained by this method, to total sulphate sulphur, as found by Folin's method, it need only be stated that out of several hundred determinations in which total sulphur and total sulphate sulphur were determined upon the same samples of urine, never once have the results for total sulphur, as determined by the above method, failed to be reasonably higher than for total sulphate sulphur.

In order to determine whether loss of any form of sulphur occurs during the carrying out of the method suggested in this paper, the entire process has been carried out in a Kjeldahl distillation flask, the evolved vapors being led through a Kjeldahl bulb (placed as for an ordinary distillation), into an excess of pure fuming nitric acid. After the heating and subsequent solution in hydrochloric acid had been effected in the flask, the fuming nitric acid contained in the receiver was evaporated to dryness (after addition of one gram of potassium nitrate), the residue heated

to fusion, dissolved in dilute hydrochloric acid, and the solution filtered. This filtrate was warmed, treated with barium chloride and allowed to stand three hours. Out of six trials, not once did a turbidity develop in these tubes.

Numerous determinations carried out upon pure solutions of sodium sulphate according to the procedure employed in the total suphur method yielded results agreeing exactly with those obtained from the pure solution alone, to which had been added 1 or 2 grams of pure sodium chloride.[1] A single example will suffice. Ten cubic centimeters of a pure sodium sulphate solution to which was added 2 grams of sodium chloride yielded (*a*) 0.0250 gm. $BaSO_4$, (*b*) 0.0254 gm. $BaSO_4$. Ten cubic centimeters of this same solution substituted for urine in the method described above gave (*a*) 0.0256 gm. $BaSO_4$, (*b*) 0.0252 gm. $BaSO_4$.

Several experiments have been carried out to determine whether there is any appreciable difference in the quantity of barium sulphate obtained after one, twenty-four, and forty-eight hours subsequent to the addition of barium chloride in the writer's process. The following results may be cited in this connection to show that such is probably not the case.

Sample	No. of hours between addition of $BaCl_2$ and filtering.	Weight of $BaSO_4$ obtained. *Grams.*
A	1	0.0705
A	1	0.0700
A	24	0.0701
A	24	0.0706
A	48	0.0706
A	48	0.0711
B	1	0.0448
B	1	0.0447
B	48	0.0445
B	48	0.0451

Finally, it was believed that it would be of interest to compare results obtained by the writer's process with those obtained by Folin's fusion method upon the same sample of urine. The following are figures of all the comparisons thus far carried out for the two methods. The Folin determinations were all made with 20

[1] I have found that very dilute sulphate solutions may yield results slightly too low, unless a considerable quantity of an electrolyte be present.

cc. of the urine, while in those carried out by the writer's process 10 cc. of the urine were employed.

No. of Sample.	Method.	Weight of $BaSO_4$ in 10 cc. urine. *Gram.*	Difference by two methods. (Folin as standard.) Gm. $BaSO_4$
1	Folin	0.0439	
1	"	0.0443	
			+0.0007
1	Writer	0.0445	
1	"	0.0451	
2	Folin	0.0625	
2	"	0.0626	
			−0.0018
2	Writer	0.0610	
2	"	0.0605	
3	Folin	0.0560	
3	"	0.0566	
			+0.0014
3	Writer	0.0580	
3	"	0.0574	
4	Folin	0.0268	
4	"	0.0266	
			+0.0002
4	Writer	0.0270	
4	"	0.0268	
5	Folin	0.0344	
5	"	0.0346	
			+0.0002
5	Writer	0.0351	
5	"	0.0344	
6	Folin	0.0385	
6	"	0.0393	
			+0.0001
6	Writer	0.0390	
6	"	0.0391	

It will be noted in the above table that plus and minus variations occur for each method, and that while in samples 2 and 3 the difference in the results is somewhat large from a percentage

point of view, it seems upon the whole that the agreement is as close as could reasonably be expected of two different methods.

It is the hope of the writer to investigate the application of this process of ashing urine to the determination of certain other urinary constituents.

In conclusion I desire to express my indebtedness to Dr. G. Edward Gage, now Associate Biologist at the Maryland Agricultural Experiment Station, for his kindness and efficiency in carrying out much detailed work in connection with the method suggested in this paper.

ADDENDUM.

It is very desirable that a new method should be checked by trials upon a substance of known purity. Through the kindness and courtesy of Prof. Otto Folin, to whom I am greatly indebted for placing his laboratory at my disposal and supplying me with a sample of cystin of exceptional purity, I am enabled to add the following data for the determination of the sulphur content of pure cystin by the copper nitrate method suggested above. 0.5 gram of chemically pure cystin was dissolved in about 10 cc. of approximately normal hydrochloric acid and the solution diluted to exactly 100 cc. Ten cc. of this solution should, theoretically, yield 0.0972 gram $BaSO_4$.

Ten cc. of the cystin solution was treated with 5 cc. of the copper nitrate potassium chlorate reagent[1] described above, and the subsequent procedure followed just as described for the urine.[2] The results of these determinations were (1) 0.0986, (2) 0.0982, (3) 0.0972, (4) 0.0984 (5), 0.0984, (6) 0.0990 gram $BaSO_4$: average, 0.0983 gram $BaSO_4$: theoretical, 0.0972 gram $BaSO_4$.

The agreement between the found and theoretical $BaSO_4$ appears to be within the limits of the experimental conditions of this work and these results add further experimental evidence in favor of the correctness of the method.

[1] This reagent gave a slight turbidity with $BaCl_2$, but the quantity of $BaSO_4$ obtained was too small to be weighed with accuracy.

[2] A few drops of sugar solution should be added to facilitate evaporation without spattering.

ON THE DECOMPOSITION OF β-OXYBUTYRIC ACID AND ACETO-ACETIC ACID BY ENZYMES OF THE LIVER.

By A. J. WAKEMAN and H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

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There is one respect in which almost all workers who have attacked the problem of fatty acid catabolism are agreed. The hypothesis that the higher fatty acids when undergoing decomposition in the animal body may part successively with groups containing two carbon atoms with resulting formation of lower fatty acids is practically unanimously accepted.[1] This series of changes in the case of a typical higher fatty acid such as palmitic acid, results eventually in the formation of butyric acid which is oxidized to β-oxybutyric acid and this in turn is further oxidized to aceto-acetic acid.

The occurrence of this latter change, namely the formation of aceto-acetic acid by the oxidation of β-oxybutyric acid, has been strikingly demonstrated by Embden and Engel,[2] who perfused surviving livers with blood containing β-oxybutyric acid or substances that under the conditions of the experiment gave rise to this substance.

It is unfortunately true that but little is known of any of the oxidizing enzymes which play a significant part in animal metabolism. With the exception of the purin derivatives almost all the substances undergoing active metabolism in the body are unattacked by the known oxidases. It was therefore particularly desirable to try to determine whether the reaction involving the oxidation of β-oxybutyric acid to aceto-acetic acid was brought about by enzymes and moreover, since the formation of even minute quantities of the latter substance could be readily followed quantitatively, the problem was well adapted to experimental investigation.

[1] Knoop: Hofmeister's *Beiträge*, vi, p. 150.

[2] *Ibid.*, xi, p. 323.

The earlier experiments were made by incubating weighed portions of minced dog's liver to which a dilute solution of ammonium β-oxybutyrate had been added. The digestion was carried out in large stoppered bottles[1] which were frequently shaken in order to secure some aëration, and care was taken to make the interval as short as possible between the removal of the liver from the body and the commencement of the incubation. The amount of aceto-acetic acid formed was estimated by distilling the incubated material and estimating the amount of acetone present in the distillate. Appropriate blank experiments were always carried out in which liver tissue and ammonium β-oxybutyrate were separately subjected to the same procedures adopted in the other cases. It was easily shown that under the conditions outlined a not inconsiderable amount of β-oxybutyric acid could be oxidized to aceto-acetic acid. The estimated amounts of the latter substance must be considerably less than the actual amount formed, since the aceto-acetic acid itself undergoes decomposition in contact with liver cells, with formation of substances other than acetone. Under favorable conditions as much as 60 milligrams of aceto-acetic acid may be obtained by the digestion of 100 grams of liver with 0.6 gram of β-oxybutyric acid.

After the oxidation had been shown to occur under these conditions, experiments were tried with aqueous extracts of the liver which were carefully freed from liver cells. The effects of the addition of a number of foreign substances was also studied.

The results of the experiments have clearly shown that the reaction is brought about by an enzyme whose action may be readily demonstrated in the absence of liver cells. The enzyme is present in aqueous extracts of liver tissue after careful filtration and centrifuging, and it may be precipitated by means of ammonium sulphate from such solutions. On dissolving the precipitate in water an active solution of the enzyme is readily obtained. The activity of the enzyme is readily abolished by heating. Employing the customary nomenclature, the enzyme may be appropriately named "β-oxybutyrase."

[1] In some experiments, in which the incubation was carried out in flasks in which the air space was small, but little action was observable.

It is an interesting fact that the action of the enzyme is markedly increased by the addition of blood, and it was found that a similar increase could be obtained by the addition of crystallized oxyhæmoglobin. It is natural to conclude that the oxyhæmoglobin acts in this way by furnishing oxygen in a form readily available for the enzyme oxidation. It is improbable, however, that oxyhæmoglobin is the only substance in the blood which exerts a beneficial action, since some acceleration of the reaction was found to be caused by blood serum.[1]

A number of experiments were undertaken with the object of determining some of the other factors influencing the course of the reaction. It was found that acetic acid (0.2 per cent) markedly inhibited the reaction while small amounts of sodium carbonate (0.1 – 0.25 per cent) appear in most cases to act favorably; but a limit is soon reached beyond which further addition of alkali exerts a decidedly harmful action.

It was found that the condition of the dog before the removal of the liver influenced the results but little. Experiments were made in which the animal had previously been starved, or given an excess of carbohydrates, or rendered diabetic either by phlorizin or by removal of the pancreas, but the results obtained did not differ materially from those obtained with normal animals.

Addition to the enzyme solution of either leucocytes, ground pancreas or spleen or bone-marrow was without constant effects, although some variations were noted. It may fairly be questioned if the type of experiment employed was adapted for detecting minute changes due to addition of foreign substances, since the amount of aceto-acetic acid produced at any given time is at best comparatively small and moreover it is constantly undergoing further decomposition.

Experiments were also made with the object of investigating the action of liver tissue and extracts upon sodium aceto-acetate. It is frequently assumed that in the body aceto-acetic acid simply undergoes decomposition into acetone and carbon dioxide but,

[1] It is probable that the conversion of butyric acid into β-oxybutyric acid is effected by a different ferment than that which effects the oxidation of β-oxybutyric acid, since under the conditions of the present experiments no appreciable increase in aceto-acetic acid resulted from the addition of ammonium butyrate to liver pulp.

although this reaction undoubtedly occurs, it is improbable that this change represents the only course of catabolism. Leathes in his *Problems of Metabolism* clearly pointed out the probability of an alternative mode of decomposition and more recently Embden and Michaud[1] have shown that the liver and other organs possess the property of decomposing significant amounts of aceto-acetic acid with formation of substances other than acetone. Embden and Michaud considered it probable that the reaction in question was not an oxidation and inclined to the view that it was probably an "acid hydrolysis," but they were unable to demonstrate any increased formation of acetic acid. The probability of this type of reaction taking place is increased by the observation of the excretion of hippuric acid following the administration of benzyl-aceto-acetic ester to a dog.[2] This result is readily understood if acid hydrolysis is assumed to occur. This would result in the formation of phenyl-propionic acid which, as is well known, is converted in the body into hippuric acid:

$$CH_3.CO.\underset{\displaystyle CH_2}{\underset{|}{\underset{\displaystyle C_6H_5}{\underset{|}{CH}}}}.COOH + H_2O = CH_3.COOH + C_6H_5.CH_2.CH_2.COOH$$

If ketone formation had taken place, analogous to the formation of acetone from aceto-acetic acid, phenaceturic acid would have been obtained in place of hippuric acid.

Our experiments upon the action of liver tissue upon the sodium salt of aceto-acetic acid confirm Embden and Michaud's results. Fresh liver tissue was found capable of decomposing considerable amounts of the aceto-acetate. It was found, in contrast to the results with β-oxybutyric acid, that addition of blood was followed by no increase in decomposition, and this observation harmonizes with the view that the reaction in question is not an oxidation. Attempts to detect the formation of acetic acid as a product of the reaction by distilling the digestion mixtures with phosphoric acid gave negative results. It was found that very much larger amounts of acetic acid could be obtained by

[1] Hofmeister's *Beiträge*, xi, p. 332.

[2] This *Journal*, vi, p. 221.

distillation after the liver tissue had been completely hydrolyzed by prolonged boiling with 30 per cent sulphuric acid. The unchanged aceto-acetic acid was removed by boiling before effecting the hydrolysis with sulphuric acid. In several cases it has been possible to demonstrate the increased formation of acetic acid in the experiments in which sodium aceto-acetate had been added, but further experiments are necessary before this point can be considered settled. The strikingly large amounts of acetic acid obtainable by the method employed clearly show that some such procedure as that involving hydrolysis of the tissue is necessary in order to liberate any acetic acid that may be formed.

EXPERIMENTAL.

The results recorded in Table I were obtained from experiments planned to demonstrate the effect of normal liver tissue, in the absence and also in the presence of blood, upon a dilute solution of ammonium β-oxybutyrate. Since all the experiments were carried out in essentially the same manner, it will suffice to indicate the general lines of procedure rather than to separately describe each individual experiment.

The ammonium β-oxybutyrate was prepared by exactly neutralizing with dilute aqueous ammonia a syrupy solution containing 68 per cent of the anhydrous β-oxybutyric acid. In all the experiments recorded in this paper with the exception of those described under Experiment XI, the amount of oxy-acid taken for each test was equivalent to 0.68 gram. The dilute ammonium β-oxybutyrate solutions were repeatedly incubated and subjected in every way to the same procedure as was adopted in the actual experiments with liver tissue. It was found that the distillates from these "blank" determinations, combined with from 0.8 to 1.0 cc. of decinormal iodine solution, equivalent to 1.5 milligrams of aceto-acetic acid.

In all the following experiments the livers of dogs were employed. The animal was lightly anæsthetized and bled from the carotid artery. In some cases (Experiments I, II, X, XII, XIII, XIV, etc.) blood was practically completely removed by perfusion with large quantities of warm salt solution. The liver was taken from the animal with as little delay as possible,

the gall-bladder removed and, after weighing, the organ was put through a previously warmed mincing machine. The pulp was rapidly divided into the requisite number of weighed portions which were transferred to warm bottles of about 2.5 liters capacity containing either ammonium β-oxybutyrate solution or salt solution previously heated to 37°. The volume of the mixture ordinarily amounted to between 200 cc. and 300 cc. and the amount of liver commonly varied from 50 to 120 grams. Warm defibrinated blood (37°) obtained from the preliminary bleeding was added when necessary. The bottles were at once placed in an incubator at 37° and the contents, which amounted to little more than one-tenth of the capacity of the bottle, were frequently shaken vigorously so as to insure some degree of aëration.[1] In a well-conducted experiment the incubation of the various mixtures had commenced within ten minutes of the death of the animal and during this interval the temperature changes were but slight.

In the majority of experiments the mixtures were incubated for five hours and at the end of that time the action was checked by the addition of 10 cc. of 20 per cent phosphoric acid. The aceto-acetic acid formed together with any acetone derived from it was estimated as follows: The incubated mixture was shaken with water and then transferred to a large flask and distilled, using an ice-cooled receiver connected with a wash-bottle containing water. The distillate was subjected to two more distillations in the usual way, first with the addition of calcium carbonate and, secondly, with a little dilute sulphuric acid. The acetone in the final distillate was estimated according to the customary method with iodine and sodium thiosulphate.

The results contained in Table I clearly show that fresh liver tissue when incubated with ammonium β-oxybutyrate solution under the foregoing conditions can bring about a marked formation of aceto-acetic acid. The reaction takes place even if the liver be freed as far as practicable from blood (Experiments I, II, X, etc.) but the action is markedly increased by the addi-

[1] In some experiments in which small flasks were used, the amount of aceto-acetic acid formed amounted to only 11 milligrams. Precisely similar experiments in which large bottles with a considerable air space were employed gave more than 50 milligrams of aceto-acetic acid.

TABLE I.

The Action of Normal Liver Tissue upon Ammonium β-Oxybutyrate in Presence and Absence of Blood.

NUMBER OF EXPERIMENT.	WEIGHT OF LIVER IN GRAMS	WEIGHT OF β-OXYBUTYRIC ACID.	BLOOD ADDED.	TIME OF INCUBATION.	MILLIGRAMS ACETO-ACETIC ACID FORMED.	NOTES.
I	100	0.68	—	5 hours	**15.3**	Liver washed free from blood by perfusion with salt solution.
	100	0.68	50 cc.	"	**22.8**	
	100	—	50 cc.	"	6.0	
	—	0.68	—	"	1.5	
II	100	0.68	—	5 hours	**10.9**	Liver washed free from blood by perfusion with salt solution.
	100	0.68	Washed cells from 70cc. blood	"	**22.3**	
	100	0.68	70cc. serum	"	**18.9**	
	100	—	—	"	4.0	
	—	0.68	Washed cells from 70cc. blood	"	4.4	
	—	0.68	70cc. serum	"	2.0	
III	115	0.68	50 cc.	5 hours	**58.6**	Liver used direct with adhering blood.
	115	—	50 cc.	"	7.8	
IV	100	0.68	50 cc.	5 hours	**45.9**	Liver used direct without removal of blood.
	100	0.68	50 cc.	"	7.8	
V	122	0.68	50 cc.	5 hours	**20.6**	"
	122	0.68	50 cc.	"	8.8	
VI	65	0.68	40 cc.	5 hours	**42.1**	"
	65	0.68	40 cc.	"	5.0	
VII	100	0.68	50 cc.	5 hours	**43.2**	"
	100	0.68	50 cc.	"	9.6	
VIII	60	0.68	60 cc.	5 hours	**32.1**	Dog starved 8 days.
	60	—	60 cc.	"	3.1	
IX	80	0.68	50 cc.	5 hours	**29.7**	Dog fed on potatoes 15 days before experiment.
	80	0.68	50 cc.	"	6.7	

TABLE I—Continued.

NUMBER OF EXPERIMENT.	WEIGHT OF LIVER IN GRAMS	WEIGHT OF β-OXYBUTYRIC ACID.	BLOOD ADDED.	TIME OF INCUBATION.	MILLIGRAMS ACETO-ACETIC ACID FORMED.	NOTES.
X	44	0.68		5 hours	11.9	Liver washed free from blood by perfusion with salt solution
	44	0.68	30 cc.	"	27.0	
	44	0.68	30 cc. +0.1% Na_2CO_3	"	27.4	
	44	0.68	30 cc. blood heated to 100°	"	17.3	
	44	—	30 cc.	"	4.5	
XI	35	0.68	20 cc.	0.5 hour	12.3	Liver used direct without removal of blood.
	35	0.68	20 cc.	1 hour	15.5	
	35	0.68	20 cc.	3 hours	17.0	
	35	0.68	20 cc.	5 hours	19.7	
	35	0.68	20 cc.	18 hours	27.9	
	35	0.068	20 cc.	5 hours	6.4	
	35	0.32	20 cc.	5 hours	10.0	

tion of defibrinated blood. If the blood be previously heated to 100° the increase is not so marked (Experiment X). The results of Experiment XI indicate the dependence of the yield of aceto-acetic acid upon the time of incubation and the concentration of the β-oxybutyrate. Experiments VIII and IX were made upon dogs one of which had starved for eight days while the other had received an excessive carbohydrate diet given in the form of potatoes. The results are not materially different from those obtained with the livers of normal animals.

The results contained in Table II indicate clearly the favorable effect upon aceto-acetic acid formation of the addition of oxyhæmoglobin. Experiment XIII shows that the addition of crystallized oxyhæmoglobin may bring about an increase in oxidation fully equal to that caused by an equivalent amount of blood. The oxyhæmoglobin crystals used in this work were prepared from dog's blood.

Table III contains a number of results showing the effect of the addition of small quantities of acid or alkali to the liver pulp. The action of the smaller amounts of sodium carbonate (0.1 to

TABLE II.

The Effect of the Addition of Oxyhæmoglobin.

NUMBER	WEIGHT OF LIVER IN GRAMS	WEIGHT OF β-OXYBUTYRIC ACID.	ADDED SUBSTANCE.	TIME OF INCUBATION.	MILLIGRAMS ACETO-ACETIC ACID FORMED.	NOTES.
XII	76	0.68	—	5 hours	**11.1**	
	76	0.68	10 grams oxyhæmoglobin	5 hours	**16.4**	The liver was washed free from blood by perfusion with salt solution.
	76	—	10 grams oxyhæmoglobin	5 hours	5.5	
	76	0.68	60 cc. blood	5 hours	**18.7**	
	—	—	60 cc. blood	5 hours	2.6	
XIII	75	0.68	—	6.2 bours	**14.9**	
	75	0.68	oxyhæmoglobin equivalent to 60 cc. blood	6.2 hours	**28.4**	The liver was washed free from blood by perfusion with salt solution.
	—	0.68	oxyhæmoglobin equivalent to 60 cc. blood.	6.2 hours	4.9	
	—	—	oxyhæmoglobin equivalent to 60 cc. blood	6.2 hours	2.0	
	75	0.68	60 cc. blood	6.2 hours	25.5	
	75	—	60 cc. blood	6.2 hours	6.1	

0.25 per cent) appears to be decidedly favorable. Larger amounts of alkali and also small amounts of acid exert a decidedly harmful effect.

In Table IV are recorded the results of experiments made with cell-free extracts of liver tissue, with precipitates obtained from these extracts by the use of ammonium sulphate and with washed liver cells which had been previously extracted with salt solution. It is clear that all of these materials can bring about the oxidation of β-oxybutyric acid in the presence of oxygen. The conclusion that this change involving the oxidation of β-oxybutyric acid to aceto-acetic acid is essentially a ferment action appears amply justified by the marked activity shown by a suspension in water of the precipitate obtained by precipitating a cell-free extract of liver tissue with ammonium sulphate. The activity of such a solution is completely destroyed by heating.

TABLE III.

The Effect of the Addition of Alkali or Acid.

NUMBER.	WEIGHT OF LIVER IN GRAMS.	WEIGHT OF β-OXYBUTYRIC ACID.	ADDED SUBSTANCE.	TIME OF INCUBATION.	MILLIGRAMS ACETO-ACETIC ACID FORMED.	NOTES.
XIV	44	0.68	—	5 hours	**11.9**	The liver was washed free from blood by perfusion with salt solution.
	44	0.68	0.1% Na_2CO_3	5 hours	**17.0**	
	44	0.68	0.3% Na_2CO_3	5 hours	**13.6**	
	44	0.68	0.6% Na_2CO_3	5 hours	9.8	
	44	0.68	1.0% Na_2CO_3	5 hours	5.8	
	44	—	—	5 hours	4.0	
XV	75	0.68	50 cc. blood	5 hours	**23.8**	
	75	0.68	50 cc. blood 0.25% Na_2CO_3	5 hours	**34.5**	
	75	0.68	50 cc. blood 0.2% Acetic acid		**8.3**	
	75	1.0 g. Butyric Acid	50 cc. blood		7.0	
	75	—			**7.0**	

The extracts were prepared by shaking the minced liver prepared as in previous experiments with a small amount of salt solution and then pressing the whole by means of a filter-press using three layers of stout filter paper. The crude extracts thus obtained were rapidly centrifuged and then in some cases filtered with the aid of suction through a thick layer of fine sand resting upon filter-paper. All these operations were carried out as rapidly as possible, avoiding any marked temperature changes. The filtrates thus obtained slowly deposit a fine protein precipitate, but upon microscopical examination no liver cells could be detected. In those experiments in which the extract was precipitated with ammonium sulphate, the salt was added in an amount slightly less than that needed for complete saturation. The precipitate was filtered off and thoroughly pressed between layers of filter-paper and then shaken with salt solution.

TABLE IV.

The action of cell-free liver extracts; of washed crushed liver cells; and of precipitates from extracts obtained with $(NH_4)_2SO_4$:

NUMBER OF EXPERIMENT.	SOURCE OF ENZYME.	β-OXYBUTYRIC ACID.	BLOOD ADDED.	TIME OF INCUBATION.	MILLIGRAMS ACETO-ACETIC ACID FORMED.	NOTES.
XVI	Extract from 110 gm. Liver	0.68	75 cc.	5 hours	**40.5**	
	Extract from 100 gm. Liver	—	75 cc.	5 hours	6.6	
	Washed cells from 110 gm. Liver	0.68	75 cc.	5 hours	**41.3**	
	Washed cells from 110 gm. Liver	—	75 cc.	5 hours	6.0	
XVII	Extract from 100 gm. Liver.	0.68	—	5 hours	**11.7**	The liver was freed from blood by perfusion with salt solution.
	Extract from 100 gm. Liver.	0.68	50 cc.	5 hours	**25.7**	
	Extract from 100 gm. Liver.	—	"	5 hours	7.2	
	Washed cells from 100 gm. Liver	0.68	0	5 hours	**10.9**	
	Washed cells from 100 gm. Liver	0.68	50 cc.	5 hours	**26.7**	
	Washed cells from 100 gm. Liver	—	"	5 hours	5.8	
XVIII	Suspension of $(NH_4)S_2O_4$ ppt. from Extract of 100 gm. Liver	0.68	50 cc.	5 hours	**24.7**	
	Suspension of $(NH_4)_2SO_4$ ppt. from Extract of 100 gm. Liver	—	50 cc.	5 hours	1.8	

TABLE IV—Continued.

NUMBER OF EXPERIMENT.	SOURCE OF ENZYME.	β-OXYBUTYRIC ACID.	BLOOD ADDED.	TIME OF INCUBATION.	MILLIGRAMS ACETO-ACETIC ACID FORMED.	NOTES.
XVIII	Washed cells from 100 gm. Liver	0.68	50 cc.	5 hours	**20.4**	
	Washed cells from 100 gm. Liver	—	50 cc.	5 hours	5.9	
	Extract from 60 gm. Liver	0.68	50 cc.	5 hours	**16.0**	
	Boiled Extract from 60 gm. Liver	0.68	50 cc.	5 hours	5.7	
XIX	Extract from 60 gm. Liver	0.68	Washed blood cells equivalent to 100 cc. blood	5 hours	**19.7**	
	Extract from 60 gm. Liver	0.68	200 cc. serum	5 hours	**14.1**	
	Washed cells from 60 gm. Liver	0.68	30 cc. blood	5 hours	**16.8**	
	Washed Cells from 60 gm. Liver	—	30 cc. blood	5 hours	5.6	
	Washed cells from 60 gm. Liver heated to 100°	0.68	30 cc. blood	5 hours	4.4	
XX	Extract from 110 gm. Liver	0.68	Washed cells from 60 cc. blood	5 hours	**20.3**	
	$(NH_4)_2SO_4$ ppt. from Extract of 110 gm. Liver	0.68	Washed cells from 60 cc. blood	5 hours	**19.4**	
	Extract from 110 gm. Liver	0.68	Serum from 60 cc. blood	5 hours	**14.8**	

TABLE IV—Continued.

NUMBER OF EXPERIMENT.	SOURCE OF ENZYME.	β-OXYBUTYRIC ACID.	BLOOD ADDED.	TIME OF INCUBATION.	MILLIGRAMS ACETOACETIC ACID FORMED.	NOTES.
XX	Extract from 110 gm. Liver	0.68	—	5 hours	**10.9**	
	Extract from 110 gm. Liver	—	—	5 hours	7.0	
	Washed cells from 110 gm. Liver	0.68	Washed cells from 60 cc. blood	5 hours	**22.3**	
	Washed cells from 110 gm. Liver	0.68	Serum from 60 cc. blood	5 hours	**18.8**	
	Washed cells from 110 gm. Liver	0.68	—	5 hours	**10.8**	
	—	0.68	Washed cells from 60 cc. blood	5 hours	4.4	
	—	0.68	Serum from 60 cc. blood		2.0	
XXI	Extract from 200 gm. Liver	0.68	50 cc.	5 hours	**20.8**	
	$(NH_4)_2SO_4$ ppt. from Extract of 200 gm. Liver	0.68	50 cc.	5 hours	**19.9**	The extract was first filtered and then centrifuged.
	$(NH_4)_2SO_4$ ppt. from Extract of 200 gm. Liver	—	50 cc.	5 hours	2.0	

TABLE V.

Experiments with the Livers of Diabetic Dogs.

NUMBER OF EXPERIMENT.	WEIGHT OF LIVER IN GRAMS.	WEIGHT OF β-OXYBUTYRIC ACID.	BLOOD ADDED.	TIME OF INCUBATION.	MILLIGRAMS ACETO-ACETIC ACID FORMED.	NOTES.
XXI	88	0.68	50 cc.	5 hours	**27.2**	Pancreas and portion of duodenum removed 1 week before expt. Last urine contained 5½ % sugar.
	88	—	50 cc.	5 hours	8.0	
XXII	90	0.68	50 cc.	5 hours	**33.6**	Pancreas extirpated 1 week previously.
	90+1.0 gram leucocytes	0.68	50 cc.	5 hours	**31.6**	
	90	—	50 cc.	5 hours	7.0	
XXIII	75	0.68	20 cc.	5 hours	**31.9**	Pancreas, spleen and part of duodenum removed 48 hours previously.
	75	—	20 cc.	5 hours	10.2	
XXIV	65	0.68	40 cc.	5 hours	**42.0**	
	65 gm. + 13 gram pancreas	0.68	40 cc.	5 hours	**32.5**	
	65 gm. + 13 gm. Pancreas + 10g. cells from duodenal mucosa	0.68	40 cc.	5 hours	**33.7**	
XXV	100	0.68	50 cc.	5 hours	**44.6**	Dog under influence of phlorizin.
	100 + 25 gm. spleen	0.68	50 cc.	5 hours	**43.6**	
	100 + 0.2 gm. leucocytes.	0.68	50 cc.	5 hours	**53.4**	
	100	—	50 cc.	3 hours	10.0	
	75	0.68	50 cc.	5 hours	**40.8**	Dog under influence of phlorizin. Last urine contained 11.7% sugar.
	75 + 0.2 gm. leucocytes	0.68	50 cc.	5 hours	**40.3**	
	75 + bone marrow from both femurs.	0.68	50 cc.	5 hours	**47.6**	
	75	0.68	50 cc.	5 hours	10.0	

TABLE VI.

The Action of Liver Tissue and Extracts upon Sodium Aceto-acetate.

WEIGHT OF LIVER USED.	TIME OF INCUBATION.	BLOOD ADDED.	ACETO-ACETIC ACID ADDED.	ACETO-ACETIC ACID LEFT UNCHANGED.	ACETO-ACETIC ACID DECOMPOSED.	NOTES.
Grams.	*Hours.*					
100	3	—	—	0.0057	—	Liver used direct with adhering blood.
100	3	—	0.0745	0.0221	0.0524	
100	3	—	0.1490	0.0561	0.0929	
100	3	—	0.2235	0.0828	0.1407	
100	3	—	0.3725	0.2230	0.1495	
100	3	—	0.1490	0.1523	—	Heated to 100°.
100	3	—	0.1490	0.1502	—	Heated to 100°.
50	20	35	0.0869	0.0178	0.0691	Liver used with adhering blood.
Cell-free Extract from 60 gm. Liver.....	20	35	0.0869	0.0979	—	No action.
$(NH_4)_2SO_4$ ppt. from extract of 60 gm. liver Liver cells....	20	35	0.0869	0.0815	0.0054	Practically no action.
60 gm. washed Liver cells....	20	—	0.0869	0.0163	0.0706	
60 gm. washed Liver cells....	20	35	0.0869	0.0184	0.0685	
60 gm. washed Liver Cells ...	20	35	—	0.088	—	
Extract from 200 gm. Liver	8	—	0.0869	0.0991	—	No action.
Extract from 200 gm. Liver	8	30 cc.	0.0869	0.1008	—	
120 gm. washed Extracted Liver cells...	9	—	0.6503	0.5062	0.1441	
120 gm.....	9	—	1.5784	1.3994	0.1790	Liver used direct with adhering blood
170 gm	7	—	1.2882	0.8705	0.4177	Liver used direct with adhering blood.

In Experiments XVII and XX the beneficial effect of blood upon the action of the ferment present in both extract and crushed cells is very marked.

Table V contains the results of some experiments which were made with the livers of diabetic dogs. The results exhibit no constant variation from those obtained from normal animals. The table also contains a few experiments in which additions of foreign cells were made. Here again, no marked variation from normal was observed.[1]

The experiments upon the action of liver tissue upon sodium aceto-acetate were in most respects similar to those of Embden and Michaud and the results of these authors are fully confirmed. The sodium aceto-acetate was prepared in the usual way by the saponification at a low temperature of ethyl-aceto-acetate with a slight excess of normal caustic soda. A rapid current of air was blown through the solution after the removal of unchanged ester and neutralization with hydrochloric acid. The object of this procedure was to remove any acetone that might be formed. The amount of aceto-acetic acid in the solution was estimated by distillation of the acidified solution and determination of the acetone thus formed.

The liver tissue and extracts were prepared in the same manner as in the experiments with β-oxybutyric acid. The results tabulated in Table VI show clearly that an enzyme capable of decomposing aceto-acetic acid without acetone formation is present in normal liver tissue; that the action is not increased by the addition of blood; and that the enzyme appears to be closely associated with the insoluble tissue constituents since aqueous extracts are practically without action. The enzyme is completely destroyed by heating the cells to 100°.

The results of the experiments planned to test the possible formation of acetic acid as a product of the decomposition of aceto-acetic acid are less satisfactory. The results of the estimation of the acidity of distillates obtained by the direct distillation of the incubated mixtures with phosphoric acid are not recorded, as we believe the figures possess little value. It would

[1] We are indebted to Dr. Alexis Carrel for performing the surgical operations referred to in Table V, and to Dr. Eugene L. Opie, who kindly furnished us with several preparations of leucocytes.

appear that much more drastic methods of analysis are necessary to free the volatile fatty acids from the combinations in which they presumably exist in the cells. We have employed a method which essentially consists in first removing undecomposed aceto-acetic acid by boiling and then completely hydrolyzing the remaining tissue by boiling for twelve hours with sulphuric acid (1 liver, 3 H_2SO_4, 6 H_2O) and finally distilling off the volatile acids with steam. The acids in the distillate were converted into barium salts and after concentration boiled with chromic acid to remove formic, sulphurous and other acids and then redistilled. The volatile acid obtained in this way was found to be almost pure acetic acid, and, in all the experiments thus far, we have obtained slightly more acetic acid from those samples of liver to which aceto-acetic acid had been added. The increase in acetic acid varied from 0.05 gram to 0.17 gram per 100 grams of liver tissue. The results are complicated, however, by the rapid acid formation that takes place in normal liver autolysis, and it will be necessary to make further experiments before drawing the definite conclusion that acetic acid is a product of the decomposition of aceto-acetic acid by enzymes.

THE LEUCIN FRACTION OF PROTEINS.

By P. A. LEVENE and DONALD D. VAN SLYKE.

(From the Rockefeller Institute for Medical Research, New York.)

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The present paper is intended, primarily as a contribution to the more exact methods for estimating the amino-acids from proteins, and, secondarily, to those for preparing l-leucin, d-isoleucin and d-valin from natural sources. Of the amino-acids obtained from proteins, tyrosin and glutaminic acid can be determined with a fair degree of accuracy, and the same is true of the three hexone bases and amide ammonia, as shown especially by the recent paper of Osborne, Leavenworth, and Brautlecht.[1] Of the thirteen remaining known amino-acids, a majority have been made readily accessible, and in better yields than previously possible by Fischer's well-known ester method. The far-reaching effects of Fischer's method, not only in the recent advance towards a more exact knowledge of protein chemistry, but in almost every field of biology, indicate the importance of developing quantitative accuracy in this field. The yields given by the original ester method were kept below quantitative by at least two sources of loss, the incompleteness with which the esters are extracted from the hydrolytic mixture, and the difficulties attending isolation of the separate acids from the groups in which they are obtained from the fractionated esters. The first source of loss is probably due either to incomplete esterification or to saponification of part of the esters before they can be extracted. It has been largely obviated by removing the mineral matter from the residues left after extracting the esters, and re-esterifying for a second and third extraction the amino-acids, usually nearly half the total, left in these residues. The first method used for this purpose was that of Abderhalden,[2] who changed by means of gaseous hydrochloric acid the masses of sodium hydrate and potash, used by

[1] *Amer. Journ. of Physiol.*, iii, p. 180, 1908.

[2] *Zeitschr. f. physiol. Chem.*, xxxvii, p. 484, 1903.

Fischer to free the esters, into chlorides, which were removed by repeated concentration of their alcoholic solution. A technique more convenient, and, we believe, less liable to loss has been developed in this laboratory.[1] The esters are freed for extraction from their hydrochlorides by means of barium hydrate, instead of sodium hydrate and potash, the barium being more easily handled and removed.

The esters are quite sharply separated by distillation into two chief fractions, one boiling above, the other below 90°, at less than 1 mm. pressure. The higher fraction contains glutaminic acid, phenylalanin, aspartic acid, and serin. With these the present paper is not concerned. We may simply mention that the determination of the first may be made quite accurately, of the second fairly so, while aspartic acid figures are less satisfactory, and serin not even roughly approximate. The lower boiling fraction contains the six acids, leucin, isoleucin, valin, prolin, alanin, glycocoll. Prolin can be separated from the others by means of its ready solubility in alcohol. This entire ester fraction is usually separated during distillation into at least two subfractions, the lower containing most of the alanin and glycocoll, the higher most of the valin and of the leucin isomers. All these acids are likely to be present in some proportion in each subfraction, however, so that the problem of their separation is unavoidable. The alanin and glycocoll, because of their ready solubility in water, can be separated from the three higher acids, the "leucin fraction," to a fair degree of completeness by fractional crystallization; then from each other by precipitating the glycocoll as ester hydrochloride, or more conveniently when, as usual, small amounts are present, as glycocoll picrate.[2]

The separation of leucin, isoleucin, and valin from one another with a completeness even approximate has been an unsolved problem. The three acids form isomorphous mixtures which are absolutely inseparable by crystallization, the only method in general use; and fractional crystallization of the copper salts is

[1] Levene: this *Journal.*, i, p. 4, 1905; Levene and Alsberg: *Ibid.*, ii, p. 128, 1906; Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 442, 1908; for details of method cf. next paper, p. 419.

[2] Levene: this *Journal*, i, p. 413, 1906.

equally futile.[1] Consequently in the numerous hydrolyses published from Fischer's laboratory, especially by Abderhalden, the substances were not usually separated, the entire mixture being reported as "leucin."[2] That the proportion of valin and isoleucin, particularly of the former, in the leucin so reported may have been very large in many cases is indicated by our results in the next paper.

The most thorough and systematic work on the leucin fraction has been done by F. Ehrlich,[3] who discovered isoleucin, and with Wendel first isolated and determined the rotation of natural l-leucin. Ehrlich and Wendel employed the first systematic method for separating the three acids of this fraction. They were changed to copper salts, which were extracted with methyl alcohol. The isoleucin and valin salts dissolve, leaving the leucin in the residue. To separate the isoleucin from the valin it was necessary to free the acids from the copper and racemicize the valin by heating the mixture of the two with barium hydrate solution in an autoclave. The copper salts were then regenerated and extracted with cold methyl alcohol or boiling 96 per cent ethyl alcohol, which removed the isoleucin salt and left the d-l-valin undissolved. This method requires lengthy manipulation, and gave only qualitative results. In the preliminary separation of leucin from valin and isoleucin the acids must be repeatedly regenerated and converted back into copper salts, which are again extracted with methyl alcohol in order to make the separation complete. The method also involves racemization of the d-valin, during which the d-isoleucin is partially

[1] Fischer: *Untersuchungen über Aminosäuren, Polypeptide und Proteine*, p. 642, 1906.

[2] Fischer: *loc. cit.* p. 67, "Aus der eben geschilderten Schwierigkeit, reines Leucin (besonders in der aktiven Form) aus dem Gemisch der Aminosäuren abzuscheiden, ergibt sich schon, dass von einer genauer quantitativen Bestimmung dieser Aminosäure nicht die Rede sein kann. Die Zahlen, die für Leucin in den zahlreichen, aus dem hiesigen Institut publizierten Hydrolysen von Proteinen angegeben sind, beziehen sich alle nicht auf das ganz reine Präparat, sondern vielmehr auf ein Gemisch mit Isoleucin, dem auch noch wechselnde Mengen von Aminovaleriansäure (Valin) beigemengt sein können."

[3] For references, cf. Ehrlich and Wendel: Zur Kenntnis der Leucinfraction des Eiweisses, *Biochem. Zeitschr.*, viii, p. 399, 1908.

changed to the isomeric d-allo-isoleucin. The racemization is almost certain to involve losses, and renders the method unavailable for preparation of either natural valin or d-isoleucin.

We have been able to effect a simple and quantitative separation of the two leucins from valin, the acids being regained without loss or racemization. The leucin isomers, when freed from valin, can be separated from each other without difficulty.

Separation of Leucin and Isoleucin from Valin.

In brief, leucin and isoleucin are precipitated as analytically pure $Pb(C_6H_{12}O_2N)_2$ when $\frac{M}{1}$ lead acetate, in quantity *slightly more than equivalent* to the leucin and isoleucin present, is added to the hot ammoniacal solution of the mixture. The valin is regained analytically pure from the filtrate, The proportions in which the two leucins are present are determined by the rotation of their mixture in 20 per cent hydrochloric acid; and they may be separated if desired, by extraction of their copper salts with methyl alcohol.

That the lead salt has not previously been used for the separation of leucin from valin seems more remarkable from the fact that it was employed by Strecker sixty years ago to first fix definitely the empirical formula and base-combining power of leucin.[1] This appears to have been overlooked by recent investigators. Levene[2] independently used precipitation with lead acetate to obtain pure leucin for analysis; and this method in a hydrolysis reported during the past year by us,[3] gave in one case, in which the proper conditions were struck largely by chance, a roughly quantitative separation of leucin from valin. It appeared that, under properly determined conditions, the method might afford means for more accurate analysis of the leucin fraction than has hitherto been possible. The present work was undertaken to fix these conditions.

It was found that:

(1) The lead compound precipitated is pure $Pb(C_6H_{12}O_2N)_2$, and the lead determination affords a quick and accurate test of the purity of the substance.

[1] *Annalen*, lxxii, p. 89, 1849.

[2] *Biochem. Zeitschr.*, v, 36, 1907.

[3] Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 466, 1907.

(2) From a 10 per cent solution of natural leucin in dilute ammonia, about 80 per cent of the leucin is precipitated by the addition of an equivalent of lead acetate.

(3) Two or three equivalents of lead precipitate little or no more completely than slightly over 1 equivalent.

(4) The presence of 3 per cent or more of d-valin in the solution makes the precipitation of the leucin practically quantitative. Apparently the valin exerts a salting-out effect on the lead-leucin salt, a property as unexpected as it is favorable for the separation.

(5) Valin is partially precipitated by an equivalent of lead acetate. If, however, valin and leucin are present together in hot solution, and only a small excess of lead is added above the amount required to precipitate the leucin, the precipitate consists of the pure leucin salt. The leucin salt appears because of its greater insolubility to be first thrown out of solution, the valin beginning to precipitate only when the leucin is completely removed and an appreciable excess of lead has been added besides.

(6) Ninety per cent alcohol appears the best medium for washing the precipitate. It does not precipitate impurities from the adherent mother liquors, and the lead salt is not appreciably soluble in it. If the alcohol is followed by ether, the precipitate can be dried *in vacuo* over sulphuric acid for analysis within an hour.

Although the lead salt is precipitated from an ammoniacal solution containing an excess of lead and valin, pure dilute ammonia dissolves considerable amounts of the leucin from the precipitate. Cold water is a better washing liquid, but may dissolve 2 to 4 per cent of the precipitate.

In view of the above facts the following procedure is employed for the separation.

Precipitation of Leucin as Lead Salt.

The leucin-valin mixture is carefully analyzed and the amount of the leucins present calculated from the carbon content.

$$\frac{\text{Per cent C} - 51.24}{3.68} \times 100 = \text{percent of leucin isomers in the mixture.}$$

51.24 = per cent C in valin; 54.92 = per cent C in leucin; 3.68 = 54.92 − 51.24.

The acids are pulverized, in order to facilitate subsequent solu-

tion, and suspended in 7 parts of water in a flask. A portion of the flocculent substance remains floating on the surface, but this does not interfere with subsequent operations. The water is heated to the boiling point, the flask then removed from the flame, and 1.5 cc. of concentrated aqueous ammonia added for each gram of substance. The flask is stoppered and shaken gently to dissolve the acids. If solution of the last particles takes place with difficulty the stopper may be loosened and the flask heated gently, but when the substance has been thoroughly pulverized this is usually unnecessary. When solution is complete, a measured amount of 1.1 M lead acetate solution (sp. gr., 1.2540 at 20°), 4 cc. for each gram of leucin calculated, is run slowly into the hot solution from a pipette or burette. The liquid in the flask is thoroughly stirred during the addition, in order to prevent accumulation of an excess of lead acetate in any portion of the solution, and consequent precipitation of valin. After the lead has been added the flask is stoppered and chilled in cold water. Usually an hour or more was allowed after the liquid had been cooled for precipitation to become complete, although a shorter time may be sufficient. The solution is filtered with suction on a Buchner funnel, or a Gooch crucible if small amounts are determined. The precipitate is removed as completely as possible from the flask, and pressed into a compact mass in the funnel. After the greater part of the adherent mother liquor is removed by suction, the precipitate is washed with several small portions of 90 per cent alcohol, then with ether, and dried *in vacuo* over sulphuric acid to constant weight. The purity of the salt is confirmed by lead determination.

In case the ratio of leucin : valin is greater than 2 : 1, as indicated by a carbon content of the mixture exceeding 53.7 per cent the precipitation of the leucin will fall somewhat short of quantitative, because valin is not present in sufficient concentration to throw it out of solution. In such cases a smaller proportion of lead may be taken, 3.7 cc. per gram of calculated leucin (3.48 cc. = exact equivalent) and the filtrate from the lead-leucin concentrated *in vacuo* until the valin has reached a concentration of about 10 per cent. Ammonia is then added, precipitating the remainder of the lead-leucin, which is washed and filtered as described above. If it is preferred, however, the filtrate may be

treated at once, after the first crop of lead-leucin is obtained, by the method described in the following paragraph for obtaining valin from the filtrate. The valin obtained in this case will be accompanied by some leucin. It is consequently analyzed and submitted a second time to the lead precipitation, which invariably completes the separation.

Recovery of Valin.

The valin is recovered quantitatively from the lead-leucin filtrate by precipitating the excess of lead with hydrogen sulphide, and evaporating the filtrate from the lead sulphide to dryness on a water bath. The valin is taken up in 3:1 alcohol-ether mixture, and washed with it in order to remove adherent traces of acetic acid and ammonium acetate. A slight amount of valin dissolves, but is regained by evaporating the filtrate to dryness, and taking up the slight amount of residue left with the alcohol-ether as before.

Purification of Lead-Leucin Salt.

In case, for any reason, too great an excess of lead has been employed, resulting in contamination of the lead-leucin by lead-valin which will be indicated by the high lead content of the precipitate, the latter may be purified by dissolving and reprecipitating. It is pulverized and dissolved in 5 parts of hot water plus one-fourth part (1 equivalent) of glacial acetic acid. When solution is complete the lead-leucin is reprecipitated by adding 0.5 cc. of concentrated ammonia for each gram of the salt. The precipitate after cooling, is collected and washed as previously described. As the proportion of valin, estimated from the lead content of the original precipitate, is usually small, the last portions of leucin must be regained from the filtrate by concentration *in vacuo*, or repurifying the residue as described in the second paragraph above.

Analysis of the Lead Salt.

The determination of lead in the leucin salt furnishes a quick and accurate means for testing its purity. The analysis is conveniently performed as follows: The sample, about 0.3 gram, is dissolved in 5 cc. of $\frac{N}{1}$ nitric acid in a 100 cc. beaker. No

heating is required. The lead is precipitated by addition of 5 cc. of $\frac{N}{1}$ sulphuric acid, followed by 50 cc. of absolute alcohol. (If alcohol is added before the sulphuric acid the precipitate is so fine that filtration is difficult.) The lead sulphate is obtained in beautiful granular form, which settles rapidly and may be filtered in 15 minutes. The precipitate is collected in a Gooch crucible and washed with 95 per cent alcohol acidified with sulphuric acid. The Gooch is set into an ordinary porcelain crucible, heated gently until the alcohol is driven off, then with the full heat of the burner for ten minutes. Duplicates by this method usually agree within a few hundredths of a per cent.

Properties of Lead-Leucin.

The salt is precipitated in flocculent form, and is easily packed into a porous cake on the filter, so that the adherent liquid is removed by the suction. Owing to its physical character, it may be freed from impurities with a small amount of washing liquid. When cold water was used several small portions totaling a volume less than that of the solution in which the salt was precipitated were found sufficient. The salt is practically insoluble in 90 per cent alcohol, so that when this is used as washing liquid, the volume does not need to be kept so small, although, judging from the analytical purity of the precipitates obtained, the alcohol is as efficient as water in removing the mother liquors.

The lead-leucin when dry appears light and flocculent, like free leucin. It shows no evidence of water of crystallization, and is not hygroscopic. After being freshly precipitated and washed with water, it may be completely dried *in vacuo* over night at room temperature. When washed with alcohol and ether, as mentioned before, less than an hour is required for drying.

On ignition the salt yields, not lead oxide but metallic lead in shining globules. If the ignition is performed carefully, and stopped as soon as the organic matter is removed, a yield of metallic lead only slightly above the theoretical is obtained. Repeated treatments in nitric acid and re-ignition are required to change the lead completely to lead oxide, so that analysis by lead oxide determination is not satisfactory. When the substance is burned a trace of carbon is apparently enclosed by the lead as carbon determinations usually come out several tenths below theoretical,

though performed on substances which determinations of Pb, N, and H show to be analytically pure. The sulphate method detailed above, however, leaves little to be desired in convenience or accuracy.

The leucin salt is readily soluble in dilute nitric and acetic acids, soluble slowly in about 100 parts of distilled water at room temperature.

Regeneration of Leucin from the Lead Salt.

In order to regain the free leucin quantitatively, the lead salt is dissolved in 15 to 20 parts hot water plus one-fourth part of glacial acetic acid, and freed from lead with hydrogen sulphide. The precipitate is filtered on a Buchner funnel, the filter paper being re-enforced by a thin mat of asbestos fiber. The lead sulphide is washed thoroughly with hot water. The filtrate is evaporated to dryness on a water bath or *in vacuo*, and the leucin washed with a small amount of 1:1 absolute alcohol-ether mixture to remove traces of acetic acid.

Polarimetric Determination of Leucin and Isoleucin.

Ehrlich has shown that l-leucin is not racemicized by heating with acids, and that it is obtained with full optical activity from proteins after hydrolysis with acids. We have found the same to be true of d-isoleucin as will be shown later. Consequently when the two are obtained quantitatively in analytically pure condition their proportions may be calculated from the specific rotation of the mixture. Because of the decided difference (21.8°) between the rotations of the two in 20 per cent hydrochloric acid, this method is probably more accurate than any direct separation, and is, of course, easily executed. It is found that the rotations of the isomers are strictly additive, neither affecting by its presence the rotation of the other; and consequently a simple, calculation gives the composition of a mixture of known optical activity. Ehrlich has established the specific rotation of d-isoleucin in 20 per cent hydrochloric acid as + 36.80. We, find however, that d-isoleucin obtained from proteins and purified by methods more effective than those available to Ehrlich has the slightly higher rotation + 37.4°, and employ this figure. Fischer and Warburg

have found a rotation of + 15.6° for l-leucin from the brucin salt of synthetic formyl leucin;[1] and Ehrlich and Wendel[2] have found the same value for l-leucin from hydrolyzed proteins. Using these values,[3] the composition of a mixture of specific rotation R is calculated by the formulæ:

$$\text{Per cent d-isoleucin} = 100 \times \frac{R - 15.6}{21.8}$$

$$\text{Per cent l-leucin} = 100 \times \frac{37.4 - R}{21.8}$$

Separation of Leucin from Isoleucin.

The isomers, free from valin and other impurities, can be separated from each other by extraction of their copper salts with methyl alcohol, as shown by F. Ehrlich for the leucins from beet sugar residues, and by Levene and Jacobs[4] for those obtained from hydrolyzed casein by lead precipitation. Consequently the method above detailed affords more accessible means than have previously been known for the preparation in quantity of natural valin and d-isoleucin, and simplifies that of l-leucin. A kilo of casein contains at least 87 grams of l-leucin, 67 grams of valin, and 15 grams of d-isoleucin, as we have determined by the methods outlined.

The isolation of d-isoleucin has previously been interfered with by the presence of d-valin, because the copper salts of both have nearly the same solubilities in methyl alcohol (1:55 and 1:52 respectively at room temperature), and the mixture of the two obtained when Ehrlich's method is applied to the leucin fraction is inseparable even qualitatively by former methods. This has prevented the preparation of more than minute amounts of d-isoleucin from proteins. Ehrlich's source has been the concentrated

[1] *Ber. d. deutsch. chem. Gesellsch.* xxxviii, p. 3497, 1905.

[2] *Loc. cit.*

[3] Locquin (*Bul. soc. chim. d. France*, (4), i, p. 601, 1907) reports a specific rotation of + 40.6° for d-isoleucin obtained from the brucin salt of the formyl derivative of synthetic d-l-isoleucin. Ehrlich's figure + 36.8°, which is generally accepted, agrees more closely with our own, + 37.4°. We hope to prepare a larger amount of pure material, and settle the question concerning the exact rotation.

[4] *Biochem. Zeitschr.*, p. 231, ix, 1908.

residue from beet sugar molasses, from which a mixture of the leucin isomers free from valin was obtained in yields of 1 to 2 grams per kilo.

EXPERIMENTAL.

Separation of Leucin and Isoleucin from Valin.

The following experiments demonstrate the precipitability, as lead salt, of leucin in pure solution; the effect of valin upon the precipitability of leucin; and the completeness with which valin can be separated from the leucins by the lead method. The leucin used showed a specific rotation in 20 per cent hydrochloric acid of + 17.2°, the valin of + 27.4.° The leucin gave, on analysis, 54.80 per cent C, 10.07 per cent H, instead of the theoretical 54.92 per cent C. and 9.99 per cent H. From its rotation it contains 92.5 per cent l-leucin, 7.5 per cent d-isoleucin. The valin gave 51.40 per cent C and 9.67 per cent H, instead of 51.24 per cent C and 9.47 per cent H. Both were obtained from hydrolysis of casein.

Nos. 1 and 2. Two grams of leucin dissolved in 14 cc. of water + 3 cc. aqueous ammonia, and precipitated by 7.5 cc. of 1.16 M lead acetate (14 per cent excess).

Nos. 3 and 4. Two grams of leucin plus 2 grams of valin dissolved in 24 cc. of water plus 5 cc. ammonia and precipitated by 7.5 cc. of 1.16 M lead acetate.

The solutions were all cooled in ice water, filtered in Gooch crucibles, the precipitates washed with small amounts of ice water, alcohol not having been tested previously, and dried to constant weight *in vacuo*. The theoretical amount of lead-leucin from 2 grams of leucin is 3.563 grams The following amounts were obtained:

No.	1	2	3	4
Amount Pb-leucin	2.724	2.892	3.528	3.567
Per cent precipitated	76.45	81.17	98.95	99.65

The precipitates gave the following figures on analysis:

No.	1	2	3	4
Substance	0.3552	0.2984	0.3377	0.3822
$PbSO_4$	0.2298	0.1931	0.2197	0.2485
Per cent Pb	44.18	44.19	44.43	44.45

Per cent Pb calculated for $Pb(C_6H_{12}O_2N)_2$, 44.29.

The leucin from precipitates 3 and 4 was quantitatively regenerated, as previously described, and gave without purification, the following figures on analysis:

0.1452 gram substance; 0.2916 gram CO_2; 0.1298 gram H_2O

	Calculated for $C_6H_{13}O_2N$:	Found:
C	54.92 per cent.	54.77 per cent.
H	9.99 per cent.	9.99 per cent.

The filtrates from the lead precipitates of 3 and 4 gave 1.919 and 1.920 grams of valin respectively, or 96 per cent each of the original 2 grams. As the substance was collected in Buchner funnels a slight loss was unavoidable in removing it from them. The samples were analyzed as follows:

No.	3	4
Substance	0.1448	0.1528
CO_2	0.2732	0.2888
H_2O	0.1239	0.1325
Per cent C	51.45	51.37
Per cent H	9.53	9.70

Calculated for $C_5H_{11}O_2N$; 51.24 per cent C; 9.47 per cent H.

The following experiments were performed to further test the effect of the presence of valin upon the precipitability of the leucins. The same valin was employed, but a different leucin, of specific rotation + 25.12° in 20 per cent hydrochloric acid, and, from this, containing 44.8 per cent isoleucin, 54.2 per cent leucin. It gave on analysis 55.11 per cent C, 10.02 per cent H. In each case the amino acids were dissolved in 12 cc. of water plus 2 cc. of ammonia, and precipitated with 3.75 cc. of 1.16 M lead acetate (0.45 cc. or 14 per cent above the equivalent of 1 gram of leucin). The precipitates were washed with 90 per cent alcohol. The theoretical yield is 1.781 gram Pb-leucin.

No.	1	2	3	4
Leucins	1.000	1.000	1.000	1.000
Valin	None	0.250	0.500	1.000
Pb–leucin	1.404	1.704	1.834	1.829
Per cent leucin precipitated	78.83	95.68	103.0	102.7

ANALYSES

Substance.....	0.3257	0.3054	0.3410	0.3164
$PbSO_4$.....	0.2111	0.1985	0.2209	0.2054
Per cent Pb.....	44.26	44.39	44.34	44.33

Calculated for Pb $(C_6H_{12}O_2N)_2$; 44.29 per cent Pb.

The precipitates were combined and the leucin regenerated.

Analyses: 0.1319 gm. substance; 0.2649 gm. CO_2; 0.1162 gm. H_2O.

	Calculated for $C_6H_{13}O_2N$:	Found:
C.....	54.92 per cent.	54.78 per cent.
H.....	9.99 "	9.86 "

The marked effect of even a small amount of valin is seen in the difference between Nos. 1 and 2. In No. 3 the maximum of leucin is precipitated. Here 0.500 gram of valin was dissolved in about 18.cc. of liquid, making approximately a 2.8 per cent solution of valin. Evidently leucin is precipitated completely from a 1:40 solution of valin. The fact that 3 per cent more than the theoretical amount of lead-leucin was obtained may be due to either a slight amount of lead-valin in the precipitate, or to the presence of a small amount of leucin in the valin used. The presence of so small a proportion of either acid as impurity in the other could hardly be detected with certainty by analysis, as it would make a difference of only 0.08 per cent in the Pb content, or, 0.11 per cent in the carbon of the free acid.

Solubility of Lead-Leucin.

The following experiments indicate roughly the relative solubilities of the salt in alcohol, cold water, and dilute ammonia (1-10). Two precipitates of 1.5245 and 1.6143 grams weight in Gooch crucibles were washed slowly with 25 cc. each of 96 per cent alcohol, followed by a few cc. of ether. They lost 0.002 and 0.004 gram respectively. After similar washing with distilled water at about 10°, followed by a few cc. of alcohol and ether, the losses were .110 and .098 gram. When the experiment was repeated using dilute ammonia (1 cc. of concentrated ammonia to 10 cc. water) the losses were 0.183 and 0.164 gram.

The solvent action of water and dilute ammonia was determined in the following experiments. In each case 0.500 gram of lead leucin was shaken 16 hours with 25 cc. of liquid, and the

organic nitrogen determined in 20 cc. of the filtrate. For this purpose the ammonia was driven off from the ammoniacal solution by adding a few cc. of 50 per cent sodium hydroxide, diluting, and boiling in Kjeldahl flasks. The solutions were then digested as usual with sulphuric acid. The method was checked by controls, in which the organic nitrogen in both filtrate and precipitate was determined, and found accurate. The duplicates represent separate solutions, not duplicate Kjeldahls on the same solutions.

cc. H_2O.	cc. conc. NH_4OH.	organic N in 20 cc.		grams leucin dissolved per 100 cc.	
		1	2	1	2
25	none	0.0124	0.0125	0.578	0.583
23.75	1.25	.....	0.0232	.....	1.085
22.50	2.50	0.0233	0.0236	1.089	1.105
20.00	5.00	0.0228	0.0226	1.064	1.056

These results indicate that the lead-leucin is about half as soluble in water as in dilute ammonia. Apparently the ideal condition for precipitation would involve having only enough ammonia present to neutralize the acetic acid formed by the reaction,

$$2\ HC_6H_{12}O_2N + Pb(C_2H_3O_2)_2 \rightleftarrows Pb(C_6H_{12}O_2N)_2 + 2\ HC_2H_3O_2$$

which would otherwise prevent the precipitation of the lead leucin. The presence of valin and a slight excess of lead acetate in solution reduce the solubility of the leucin salt sufficiently, however, to make this precaution appear unnecessary.

Precipitability of Other Amino Acids by Lead and Ammonia.

Three-tenths gram of each acid were dissolved in 2 cc. of hot water (except tyrosin which required several cubic centimeters) plus 0.5 cc. of concentrated ammonia, then 1 cc. of 1.16 M lead acetate added. l-Tyrosin, l-phenylalanin, d-l-phenylalanin, d-valin, and d-l-valin were precipitated at once. d-Glutaminic acid precipitated on cooling. d-l-Aspartic acid precipitated only after a second cubic centimeter of lead acetate, making nearly an

equivalent, had been added. d-Alanin, l-prolin, l-oxyprolin, and d-l-serin were not precipitable.

Tyrosin when present with leucin, is precipitated with the latter as lead salt, so that the lead method for obtaining leucin cannot be used in the presence of tyrosin.

Polarimetric Determination of Proportions of Leucin and Isoleucin in Mixture.

Ehrlich's rotations of leucin in 20 per cent hydrochloric acid have all been taken with solutions of about 3.65 per cent concentration, while the rotation of isoleucin has been uniformly taken with solutions of about 4.5 per cent strength. In order to make certain that the polarimetric method is applicable for quantitative determinations, the effect of concentration upon the specific rotation must be known.[1] The following determinations, upon a sample of analytically pure leucin obtained by the ester and lead methods from casein, show that the specific rotation of leucin is within ordinary limits, independent of the concentration. The observations were all taken with a tube of 1.894 dm. length, and the specific gravity of the solutions was 1.099, not being appreciably affected by this change in leucin concentration.

NO.	WT. LEUCIN.	TOTAL WT. SOLUTION.	PER CENT LEUCIN.	OBSERVED ROTATION.	$[\alpha]_D^{20°}$
				degrees.	*degrees.*
1	0.4201	18.225	2.304	+ .83	+17.29
2	0.5871	19.744	2.974	+1.04	+17.31
3	0.7689	22.698	3.386	+1.19	+16.87
4	0.6984	18.994	3.677	+1.32	+17.23
5	0.8808	19.140	4.603	+1.64	+17.10
6	1.0518	18.172	5.789	+2.09	+17.33

It is evident that, at least below 5.8 percent concentration, the specific rotation of leucin is practically independent of the con-

[1] W. Jones (this *Journal*, v, p. 1, 1908) has shown that the specific rotation of the nucleic acids varies markedly with the concentration. This is also true of some other substance (Vaubel, *Physik. u. chem. Methoden der quant. Best. org. Verbindungen*, i, p. 435.)

centration. The rotation of isoleucin is also constant as will be shown presently.

It remains to determine the mutual effect of leucin and isoleucin upon their rotations when in solution together. The following table shows that each exhibits its normal rotation unaffected by the other, the rotations of mixtures being additive. Neither the leucin nor the isoleucin used was entirely free from the other, but this is not necessary in order to demonstrate the simple additivity of their rotations. The leucin (A) was from the lot used in the preceding experiment. The isoleucin (B) also was analytically pure, and showed a rotation of 33.46° instead of 36.80°.

NO.	GRAMS A.	GRAMS B.	CONCENTRATION OF SOLUTION PER CENT	$[\alpha]_D^{20°}$ OBSERVED.	$[\alpha]_D^{20°}$ CALCULATED FROM AMT. A AND B.
1	Av. 6 rotations	.0000	2.3–5.8	+17.19	.....
2	.7747	.2179	4.868	+20.96	+20.75
3	.3870	.3870	4.051	+25.49	+25.35
4	.2041	.5983	4.200	+29.10	+29.36
5	.0000	.8298	4.332	+33.46	.....

The calculated and observed rotations agree within the limit of error. The calculated rotations are computed by the formula,

$$[\alpha]_D^{20°} = 17.19 + \frac{a}{a+b} \times 16.37$$

a representing the grams of A (leucin) in second column, b the grams of B (isoleucin) in the third. $16.37° = 33.46° - 17.19°$, the difference between the rotations of A and B. The degree of accuracy of the polarimetric method is indicated by the following figures from the preceding data.

NO.	PER CENT A PRESENT.	PER CENT B PRESENT.	PER CENT A CALCULATED FROM ROTATION.	PER CENT B CALCULATED FROM ROTATION.
2	78.0	22.0	76.9	23.1
3	50.0	50.0	49.1	50.9
4	25.4	74.6	27.0	73.0

The calculated percentages are by the equation:

$$\text{Per cent B} = \frac{[\alpha]_D^{20°} - 17.2}{16.37} \times 100.$$

$$\text{Per cent A} = 100 - \text{per cent B.}$$

From the above results it is evident that the rotation of a leucin-isoleucin solution is a simple linear function of the percentages of the constituents. Consequently, the percentages of the latter in a mixture of ascertained specific rotation in 20 per cent hydrochloric acid may be calculated by the equations already given.

The applicability of the above polarimetric method rests, of course, on the correctness of the assumption that l-leucin and d-isoleucin are the only leucin isomers occurring in natural proteins, and that these are not racemicized by acid hydrolysis. All the work which has been done on the natural amino-acids failed to disclose any indication of the presence of other isomers, and F. Ehrlich's[1] work on this point especially indicates the non-existence of other isomers in proteins. Ehrlich and Wendel[2] have also demonstrated conclusively that the l-leucin obtained by acid hydrolysis possesses full optical activity, and that this is not decreased in the slightest by 20 hours boiling of the pure l-leucin in 33 per cent sulphuric or concentrated hydrochloric acids. We have found the same to be true of d-isoleucin.

This was already rendered probable by the work of Weitzenboeck[3] and of Ehrlich and Wendel. Weitzenboeck obtained from casein by acid hydrolysis and esterification, isoleucin of $[\alpha]_D^{20°} = +38.3°$ in 22 per cent hydrochloric acid. The deviation from Ehrlich's figure is about what would be expected from the difference in the concentration of hydrochloric acid used as solvent. Loquin[4] found that changing the concentration of the solvent hydrochloric acid solution from 8 per cent to 12 per cent increased the specific rotation of isoleucin by 9°, an average of $\frac{3}{4}°$ for each per cent increase in hydrochloric acid. Ehrlich and Wendel obtained mixtures of isoleucin and valin from acid hydrolyses

[1] *Ber. d. deutsch. chem. Gesellsch.* xxxvii, p. 1809, 1909.
[2] *Biochem. Zeitschr.*, viii, p. 412, 1908.
[3] *Monatsh. f. Chem.*, xxvii, p. 831, 1906.
[4] *Bull. soc. chim. d. France*, (4), i, p. 160, 1907.

of casein, spongin and ovalbumin, the rotations of which varied between + 30.00° and + 32.40°, the carbon from 51.77 per cent to 53.76 per cent, nitrogen accordingly. The rotation of d-valin is + 28.8°, of d-isoleucin + 37.4°, the corresponding carbon contents being 51.24 per cent and 54.96 per cent. It is evident that the isoleucin could have lost little of its optical activity, or its presence would not have so markedly raised the rotation of the mixture above that of d-valin.

We have found that isoleucin is obtained in full optical activity from casein hydrolyzed with acid and subjected to the usual ester method; and that its rotation is not affected by 18 hours boiling with 20 per cent hydrochloric acid. As a preliminary experiment, a 1.3 gram portion of isoleucin previously prepared by Levene[1] of rotation 34.6° ± 0.3°, was boiled 18 hours with 20 per cent hydrochloric acid. The solution was then freed from hydrochloric acid by means of silver sulphate followed by hydrogen sulphide and the required amount of barium hydrate. 1.14 grams of isoleucin were regained by concentrating the solution almost to dryness, and crystallizing from alcohol.

Rotation in 20 per cent hydrochloric acid: 0.7280 gram substance; 19.212 grams total solution; per cent isoleucin, 3.790; rotation in 1.894 dm. tube, +2.76°; sp. gr. 1.099.

$$[\alpha]_D^{20°} = + 34.96° \pm 0.3°$$

In order to test the effect of concentration on the specific rotation, 9.922 grams of the above solution were diluted to 19.180 grams with 20 per cent hydrochloric acid, making a 1.96 per cent solution of isoleucin. This showed a rotation of + 1.43° in a 1.894 dm. tube.

$$[\alpha]_D^{20°} = + 35.0° \pm 0.5°$$

It appears that boiling in strong acid, such as is used for hydrolysis, does not affect the rotation of isoleucin, and that the specific rotation is, like that of l-leucin, independent of the concentration, within ordinary limits.

Preparation of Pure d-Isoleucin from Casein.

The isoleucin used for the above experiments evidently contained a small amount of l-leucin, its rotation being 2° below

[1] Levene and Jacobs: *Biochem. Zeitschr.*, ix, p. 231, 1908.

that given by Ehrlich for d-isoleucin. In order to make the test more rigid another portion of the substance was prepared, with full optical activity according to Ehrlich's figures. The source was 10 grams of leucin obtained from casein hydrolyzed with 25 per cent hydrochloric acid. The leucin was obtained by the previously described lead method from the esters boiling between 60° and 80° at 0.5 mm. It showed a specific rotation of + 22.3° in 20 per cent hydrochloric acid indicating 31 per cent of d-isoleucin, 69 per cent of l-leucin. The substance was transformed into copper salts by boiling with a small excess of copper oxide, and extracting the latter with large volumes of boiling water to remove the difficultly soluble leucin salt. The solution was concentrated to dryness *in vacuo*. The dry copper salts were ground fine and extracted 48 hours in a shaking machine with 500 cc. of 94 per cent Merck's methyl alcohol, then washed thoroughly with the same solvent. Apparently the l-leucin salt is slightly soluble, for shaking with successive portions of methyl alcohol continued dissolving slight amounts of copper salt; although the first extraction was performed with enough alcohol to take up 9 grams of isoleucin-copper. The first extraction yielded 2.9 grams of copper salt, and only this was used for isoleucin. The copper content was determined by Volhard titration.

0.1715 gram of substance required 5.40 cc. of $\frac{N}{10}$ NH_4SCN, indicating 0.0343 gram Cu or 20.02 per cent, the theoretical being 19.64 per cent Cu. The salt was decomposed with hydrogen sulphide, and the isoleucin purified by precipitating once as lead salt, then recrystallizing from dilute alcohol.

Analysis: 0.1535 gram substance; 0.3090 gram CO_2; 0.1409 gram H_2O.

	Calculated for $C_6H_{13}O_2N$:	Found:
C.....	54.92 per cent	59.90 per cent.
H	9.99 "	10.27 "

Rotation in 20 per cent hydrochloric acid: 0.6116 gram substance; 18.968 grams solution; rotation in 1.894 dm. tube, + 2.40°; sp. gr. 1.099.

$$[\alpha]_D^{20°} = + 35.76°.$$

As noted above, it appeared that a slight amount of l-leucin copper might have been dissolved, even at room temperature, by the large excess of methyl alcohol used. Consequently the isoleucin was changed back into the copper salt, which was extracted

with only 150 cc. of methyl alcohol. A portion remained undissolved. The soluble portion yielded isoleucin of the following rotation.

0.5158 gram substance; 11.372 grams total solution; concentration of solution, 4.536 per cent; sp. gr., 1.099; rotation in 0.865 dm. tube, + 1.61° ± .01°

$$[\alpha]_D^{20°} = + 37.35° \pm 0.23°$$

5.89 grams of the above solution were diluted to 11.427 grams with 20 per cent hydrochloric acid making a 2.34 per cent solution. Rotation in 0.865 dm. tube, + 0.825° ± .01°

$$[\alpha]_D^{20°} = + 37.10° \pm 0.4°$$

The isoleucin was boiled 18 hours with 20 per cent hydrochloric acid freed from hydrochloric acid as described before, and its rotation in 20 per cent hydrochloric acid repeated.

0.2224 grams substance; 11.200 grams solution; concentration of solution 1.986 per cent; sp. gr., 1.099; rotation in 0.865 dm. tube, + 0.696° ± .01°

$$[\alpha]_D^{20°} = + 36.9° \pm 0.5°$$

The above results indicate:

(1) That d-isoleucin is not racemicized by prolonged boiling with strong hydrochloric acid.

(2) That it is obtained with full optical activity from proteins hydrolyzed with acid and subjected to the ester method.

(3) That the specific rotation is the same at different concentrations.

Consequently, the same properties having been demonstrated for l-leucin, the polarimetric method may be relied upon for the determination of l-leucin and d-isoleucin obtained by acid hydrolysis of proteins.

d-Isoleucin from Edestin.

The amino acids of the leucin fraction obtained, after acid hydrolysis of edestin, partly by crystallization, partly by the ester method, were changed to copper salts, and the latter extracted with methyl alcohol. From the copper salts soluble in methyl alcohol, 13 grams of crude isoleucin-valin mixture were regained. As the substance was considerably tinged with brown, it was not subjected to preliminary analysis, but at once dissolved and precipitated with lead-equivalent to 8 grams of isoleucin.

The filtrate from the lead-isoleucin yielded 6 grams of pure recrystallized valin, giving 51.47 per cent C, 9.21 per cent H on analsis, and showing specific rotation of + 26.8° in 20 per cent hydrochloric acid.

The lead salt was decomposed, and yielded 3.6 grams of isoleucin, which, because of the large excess of lead that had been used, still contained a small amount of valin.

Analysis: 0.1226 gram substance; 0.2440 gram CO_2; 0.1111 gram H_2O.

	Calculated for $C_6H_{13}O_2N$:	Found:
C.....	54.92 per cent	54.28 per cent.
H.....	9.99 "	10.14 "

The specific rotation in 20 per cent hydrochloric acid was + 35.3,° that found by us for purified natural isoleucin from casein being + 37.35.°

To free entirely from valin, 3.2 grams of the substance were dissolved as usual and precipitated with an equivalent of lead acetate. 2.5 grams of isoleucin were regained, of the following rotation:

0.3024 gram substance; 18.731 grams solution; concentration, 1.618 per cent; sp. gr., 1.099; rotation in 1.855 dm. tube, + 1.19°.

$$[\alpha]_D^{20°} = + 36.91° \pm 0.3°$$

To complete the purification, the isoleucin was transformed again into the copper salt, 2.56 grams of which were shaken over night with 170 cc. of methyl alcohol. This was only slightly more than the amount required to dissolve the copper salt of pure isoleucin, and insufficient to take up appreciable amounts of l-leucin copper salt. All but 110 mg. of the copper salt dissolved in the methyl alcohol. The solution was concentrated to dryness, and the salt dissolved in water and decomposed with hydrogen sulphide. The isoleucin regained was passed once more through the lead process, and crystallized from a small amount of alcohol. 1.47 grams were regained.

Rotation in 20 per cent hydrochloric acid; 0.6978 gram substance; 18.732 grams solution; concentration, 3.726 per cent; sp. gr., 1.099; rotation in 1.855

dm. tube, + 2.85° and + 2.84° respectively by two observers, average, + 2.845.°

$$[\alpha]_D^{20°} = + 37.44° \pm 0.15°$$

Analysis: substance, 0.1430 gram; CO_2, 0.2876 gram; H_2O, 0.1269 gram.

	Calculated for $C_6H_{13}O_2N$:	Found:
C.....	54.92 per cent.	54.86 per cent.
H.....	9.99 "	9.93 "

The fact that careful purification, through the copper and lead salts and recrystallizing, raised the rotation only 0.5° indicates that the product is about as pure as can be obtained from natural sources by present methods. The rotation is identical with that (+ 37.35°) of the isoleucin from casein.

Separation of Leucin and Valin as Obtained from Casein.

Following is a description of the manipulation of a fraction of amino acids which consisted mainly of the equimolecular mixture of leucin and valin which defies separation by the fractional recrystallization method. The fraction was obtained from the third crop of amino acids crystallizing from several pounds of casein which had been digested six weeks with trypsin, in order to prepare amino-acids for physiological experiments. The crop of crystals referred to was esterified, the esters freed with barium hydrate, and distilled, yielding the following fractions:

Fraction	Temp. vapors. *degrees.*	Pressure. *mm.*	Weight of esters. *grams.*
I	to 70°	15	28
II	60°–80°	0.25	111
III	80°–120°	0.40	63

Fraction II contained chiefly the leucin fraction. It was separated into subfractions, with the object of obtaining mixtures of the leucins and valin in variations covering all the range likely to be met with in proteolytic products. We have shown previously that extraction of the mixed esters with ether gives an ether-soluble portion containing most of the leucin, and a water-soluble

containing most of the valin.[1] This method was combined with fractional crystallization.

The esters were poured into 3 volumes of water, and the mixture extracted with an equal volume of ether. The ethereal solution was washed six times with water, a volume of the latter equal to about two-thirds that of the ethereal solution being used for each washing. The esters contained in the water and ether respectively were saponified separately by boiling with water, and the amino-acids from the former separated into four, those from the latter into two fractions by crystallization. The leucin and valin in each were separated by the lead method. Fractions 5 and 6 were freed from prolin by boiling with alcohol. It is noteworthy that the ether-soluble esters were entirely free from prolin.

The fractions were of the following quantity and analysis:

NO.	WEIGHT IN GRAMS.	PER CENT C.	PER CENT H.	REMARKS.
1.....	37.43	54.26	9.96	From ether soluble esters
2.....	8.32	52.74	9.27	" " " "
3.....	8.13	53.74	10.09	" water " "
4.....	12.54	53.52	9.55	" " " "
5.....	7.50	52.79	9.39	" " " "
6.....	0.48	47.13	8.55	" " " "

Fraction 1. 37.43 grams, 54.26 per cent C. From the carbon content, this fraction should contain 81.5 per cent or 30.5 grams of leucin. Because of its high proportion, the leucin had to be removed in two portions (cf. p. 396). The mixture was dissolved in 250 cc. hot water and 50 cc. concentrated ammonia, and precipitated with 120 cc. $\frac{M}{1}$ lead acetate, equivalent to 31.4 grams leucin. 51.43 grams of lead-leucin were precipitated, equivalent to 28.87 grams of leucin. Analysis:

I. 0.3705 gm. substance, 0.2397 gm. $PbSO_4$

II. 0.2313 gm. " 0.2558 gm. CO_2, 0.1050 gm. H_2O.

III. (1) 0.4330 gm. " 18.45 cc. $\frac{N}{10}$ H_2SO_4 (Kjeldahl)

(2) 0.4326 gm. " 18.28 cc. $\frac{N}{10}$ " (")

[1] Levene and Van Slyke; *Biochem. Zeitschr.*, xiii, p. 442, 1908.

	Calculated for $Pb(C_6H_{12}O_2N)_2$	Found:
Pb	44.29 per cent.	44.20 per cent.
C	30.83 "	30.14* "
H	5.18 "	5.07 "
N	6.00 "	5.98 "
		6.03 "

* Carbon as a rule is found low, probably because carbon particles are enclosed in the lead-globule formed when the salt decomposes.

Twenty grams of the salt were freed from lead, and the leucin regenerated. 10.77 grams were obtained, calculated 11.22 grams. Analysis: 0.1566 gram substance; 0.3164 gram CO_2; 0.1404 gram H_2O.

	Calculated for $C_6H_{13}O_2N$:	Found:
C	54.92 per cent.	55.11 per cent.
H	9.99 "	10.02 "

Rotation in 20 per cent hydrochloric acid: 0.5072 gram substance; 11.125 grams total solution; sp. gr., 1.10; observed rotation in 0.865 dm. tube, + 1.09°.

$$[\alpha]_D^{20°} = + 25.12°$$

According to the rotation the "leucin" was at least 44.8 per cent isoleucin. As the substance was obtained from prolonged tryptic digestion in alkali, some racemization may have occurred, and the correct figure be somewhat higher.

The filtrate from the lead-leucin, freed from lead, evaporated to dryness and washed with absolute alcohol yielded 7.83 grams of a mixture of leucin and valin. The loss of 0.73 gram was not noticed until the wash alcohol had been discarded. Thereafter the wash alcohol was evaporated to dryness, and a second crop obtained, which made the recovery of the amino-acids quantitative.

The mixture gave on analysis 52.94 per cent C, 0.44 per cent H. From the carbon content the 7.83 grams should contain 46 per cent leucin or 3.60 grams, and 4.23 grams of valin. 7.64 grams were dissolved in 50 cc. of water plus 5 cc. ammonia, and precipitated with 15 cc. $\frac{M}{1}$ lead acetate. 5.84 grams of lead-leucin, equivalent to 3.28 grams of leucin were obtained.

Analysis: (1) 0.3105 gm. substance; 0.2018 gm. $PbSO_4$; 44.38 per cent Pb.
(2) 0.3413 " " ; 0.2214 " " ; 44.30 " "
Calculated for $Pb(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

The filtrate yielded 4.20 grams of valin.

Analysis: 0.1523 gram substance; 0.2859 gram CO_2; 0.1281 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.24 per cent.	51.21 per cent.
H	9.47 "	9.41 "

Rotation of valin in 20 per cent hydrochloric acid: 0.4730 gram substance; 11.220 gram total solution; sp. gr., 1.10; obtained rotation in 0.865 dm. tube, + 1.02°.

$$[\alpha]_D^{20°} = + 25.4°.$$

The rotation agrees with that found by previous observers for natural valin, although lower than the synthetic d-valin of Fischer, which showed a specific rotation of + 28.8° ± 0.4°.[1] The rotation of valin from natural sources varies through a range of several degrees below this, presumably because it is somewhat racemized. The possibility is not excluded, of course, that the variations may be due to the presence of small amounts of the low-rotating isovalin synthesized by Ehrlich, as the high rotation formerly attributed to leucin was due to the presence of small amounts of high-rotating isoleucin.[2]

The yields of leucin and valin calculated for 7.83 grams are 3.36 grams and 4.30 grams respectively, making for Fraction 1, 32.29 grams of leucin, 4.30 grams of valin, 0.84 or 2.2 per cent being lost in separation.

[1] *Ber. d. edutsch. chem. Gesellsch.*, xxxix, p. 2320, 1906.

[2] The following table shows the various rotations obtained by several authors for analytically pure samples of natural valin.

AUTHOR	SOURCE OF VALIN	ROTATION IN 20% HCl	REFERENCE
		degrees.	
E. Fischer	Casein	+27.95	*Zeitschr. f. physiol. Chem.*, xxxiii, p. 165, 1901.
Fischer and Dörpinghaus	Horn	+25.9	*Zeitschr. f. physiol. Chem.*, xxxvi, p. 469, 1902.
Schulze and Winterstein	Lupinus Luteus	+28.2	*Zeitschr. f. physiol. Chem.*, xxxv, p. 301.
	Lupinus Albus	+27.9	
Abderhalden	Edestin	+26.7	*Zeitschr. f. physiol. Chem.*, xl, p. 249, 1903.
T. B. Osborne	Phaseolin	+23.74	*Zeitschr. f. anal. Chem.*, xlviii, p. 102, 1909.
		+24.66	
	Glutinin	+25.63	

Fraction 2. 8.32 grams, 52.74 per cent C. The substance was dissolved in 50 cc. of water plus 10 cc. of ammonia, and precipitated with 20 cc. of $\frac{M}{1}$ lead acetate; 6.96 grams of lead salt were obtained, equivalent to 3.91 grams of leucin.

Analysis: 0.4161 gm. substance; 0.2690 gm. $PbSO_4$; 44.16 per cent Pb.
Calculated for Pb $(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

The filtrate yielded 4.32 grams of somewhat impure valin. The substance was recrystallized from dilute alcohol, 3.80 grams being regained of the following composition.

0.1696 gm. substance; 0.2968 gm. CO_2; 0.1416 gm. H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.24 per cent.	51.65 per cent.
H	9.47 "	9.33 "

Rotation of valin in 20 per cent hydrochloric acid: 0.6704 gram substance; 16.757 gram total solution; sp. gr., 1.10; observed in 0.865 dm. tube, + 1.02°.

$$[\alpha]_D^{20^\circ} = + 26.8^\circ$$

The mother liquors evaporated to dryness yielded 0.3 gram of mixture of valin with presumably alanin, with 49.83 per cent C, 8.73 per cent H. Fraction 2 consequently yielded 3.91 grams of leucin, 3.80 grams of valin, 0.30 gram of impure valin, 0.2 gram being lost in manipulation.

Fraction 3. 8.13 grams, 53.52 per cent C. The substance was dissolved in 50 cc. of water plus 12 cc. of ammonia, and precipitated by 21 cc. of $\frac{M}{1}$ lead acetate. 9.00 grams of lead-leucin equivalent to 5.05 grams of leucin, were obtained.

Analysis: 0.3170 gm. substance; 0.2045 gm. $PbSO_4$: 44.06 per cent Pb.
Calculated for Pb $(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

From the filtrate 2.92 grams of valin were regained.

Analysis: 0.1201 gram substance; 0.2264 gram CO_2; 0.1031 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.24 per cent.	51.41 per cent.
H	9.47 "	9.60 "

Rotation in 20 per cent hydrochloric acid; 0.4817 gram substance; 11.225 grams solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.07°.

$$[\alpha]_D^{20^\circ} = + 26.2^\circ$$

Fraction 3 yielded 5.05 grams of leucin, 2.92 grams of valin, 0.16 gram being lost in manipulation.

Fraction 4. 12.54 grams, 52.79 per cent C. The substance was dissolved in 80 cc. of water plus 20 cc. of ammonia and precipitated with 25 cc. of $\frac{M}{1}$ lead acetate, equivalent to 6.5 grams of leucin. 8.96 grams of lead-leucin, equivalent to 5.03 grams of leucin, were obtained.

Analysis: (1) 0.3323 gm. substance; 0.2153 gm. $PbSO_4$; 44.25 per cent Pb.
(2) 0.2274 " " 0.1477 " " 44.36 " "
Calculated for Pb $(C_6H_{12}O_2N)_2$; 44.29 per cent Pb.

The filtrate yielded 7.32 grams of valin.

Analysis: 0.1507 gram substance; 0.2844 gram CO_2; 0.1269 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.24 per cent	51.45 per cent.
H	9.47 "	9.42 "

Rotation of valin in 20 per cent hydrochlorate acid; 0.4972 gram substance; 11.129 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.14°.

$$[\alpha]_D^{20^\circ} = + 26.8^\circ$$

Fraction 4 yielded 5.05 grams of leucin and 7.32 grams of valin, a total of 12.37 grams from the original 12.54 grams of mixture.

Fraction 5. 7.50 grams. This entire fraction had the composition of valin.

0.1838 gram substance; 0.3440 gram CO_2; 0.1543 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.24 per cent.	51.28 per cent,
H	9.47 "	9.39 "

Fraction 6. 0.48 gram, 47.13 per cent C., 8.55 per cent H. This fraction was probably a mixture of valin and alanin. It was discarded.

The results of the above separation are collected in the following table:

FRACTION.	WEIGHT.	LEUCIN AND ISOLEUCIN.	VALIN.	TOTAL LEUCIN AND VALIN.	LOSS AND IMPURITIES.
1	37.43	32.29	4.30	36.59	0.84
2	8.32	3.91	3.80	7.71	0.61
3	8.13	5.05	2.92	7.97	0.16
4	12.54	5.03	7.32	12.35	0.19
5	7.50		7.50	7.50	0.00
Total	73.92	46.28	25.84	72.12	1.80

97.6 per cent of the total amino-acids in the five fractions was regained as analytically pure leucin and valin. But for the unnecessary loss in the first, the yield would have been somewhat higher.

SUMMARY.

l-Leucin and d-isoleucin may be separated quantitatively from d-valin by precipitation as the normal lead salt, $Pb(C_6H_{12}O_2N)_2$. The relative proportions of the leucin isomers may be determined polarimetrically by the rotation of their analytically pure mixture in 20 per cent hydrochloric acid. An exact analysis of the important leucin fraction of proteins is thus rendered possible for the first time.

For purposes of preparation the leucin isomers obtained free from valin may be separated by Ehrlich's method of extracting their copper salts with methyl alcohol. The formerly difficult preparation in quantity of natural d-valin, l-leucin, and d-isoleucin is rendered comparatively simple.

Isoleucin preparations from casein and edestin showed, after repeated purification by means of the lead and copper salts, and by recrystallization, specific rotations of + 37.35° and + 37.44° respectively in 20 per cent hydrochloric acid. The figure + 37.4° doubtless more nearly expresses the rotation of pure natural isoleucin than + 36.8°, the slightly lower figure found by Ehrlich, who had less complete means of purifiying the substance.

THE LEUCIN FRACTION IN CASEIN AND EDESTIN.

By P. A. LEVENE and DONALD D. VAN SLYKE.

(*From the Rockefeller Institute for Medical Research, New York.*)

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An accurate method having been developed for determination of the valin, leucin and isoleucin which compose the "leucin fraction" of the complex proteins, it seemed desirable for comparison to repeat the analyses of this fraction as obtained from one or more proteins which have already been subjected to hydrolysis under what might be regarded as standard conditions according to previous methods. Casein and edestin from flax seed were chosen, because they are, if not definite chemical compounds, at least obtainable by well-fixed methods which should insure products of definite character and composition; and their hydrolyses have been performed by Abderhalden, who has probably had more experience than any other worker in the field of protein hydrolyses.

Casein.

Four hundred and sixty-four grams, calculated ash and moisture free, of Kahlbaum's casein prepared according to Hammarsten, were hydrolyzed by 12 hours' boiling with 25 per cent hydrochloric acid and esterified. The esters were divided into two portions for convenience, freed with barium hydrate, and extracted. The residue was esterified a second time, and another repetition yielded a small third crop of esters.

Because of the extreme convenience of the barium method for freeing the esters,[1] we repeat in some detail the manner in which it is now used in this laboratory. The alcoholic solution of ester hydrochlorides is concentrated *in vacuo* as usual. The great part of the alcohol is driven off, without carrying the concentration so far that the solution is too thick to remove easily from the flask. Keeping the temperature of the bath at 45° to 50° towards the close of the concentration assures this. The concentrated esters are poured into an enamelled jar, of about 2.5 liters

[1] First used by Levene, this *Journal*, i, p. 4, 1905; Levene and Alsberg: *Ibid.*, ii, p. 128, and further developed by Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 442, 1908.

capacity for the products of 250 grams of protein, and the flask rinsed with a small volume of ice-cold baryta water to which is added powdered barium hydrate. The jar with esters and rinsings is placed in a freezing mixture and stirred with a wooden spatula while pulverized barium hydrate is added rapidly. The mass, at first usually thick, becomes in a few minutes semi-liquid and alkaline. As soon as the alkaline reaction appears the mixture is covered with ice-cold ether, and the stirring continued, anhydrous barium hydrate in portions of 10 to 20 grams being now added at intervals of several minutes. As the fluid water is taken up by the anhydrous baryta the esters are removed by the ether. The residual mass finally becomes sufficiently dry to break into small, easily stirred granules. They still contain enough moisture to hold them together and prevent formation of a suspension of dry powder in the ether. When the granular condition is reached the addition of barium hydrate is discontinued. If this point is passed and a suspension forms, the ether must be filtered on a large Buchner. When the anhydrous baryta is added with moderate care, however, no trouble is experienced in reaching the proper end point. The ice-cold ether is renewed several times during the operation, and the extraction continued until the extracts are colorless. The latter are shaken with potash and dried over sodium sulphate as usual.

In order to make the yield of esters as nearly quantitative as possible, the barium residue is thoroughly triturated several times with water, and finally washed on a Buchner funnel. The washings, which contain the amino-acids not removed by the first esterification and extraction, are quantitatively freed from barium by sulphuric acid, concentrated *in vacuo*, and re-esterified. Extraction as described above yields a second smaller crop of esters, and by repeating the process, a third and even a fourth crop in decreasing yields may be obtained.

The freeing of the esters is really the critical point in the ester method, and the above technique has several advantages over the usual sodium hydrate and potash method. A large excess of free alkali in solution is impossible when barium is used, because the low solubility of barium hydrate in the cold makes its concentration a self-regulating function. For the same reason, the heat of neutralization is generated no faster than it can be removed by the freezing mixture, so that over-heating is avoided and saponification of esters undoubtedly reduced. It is not difficult to keep the temperature of the mixture below 5° while the esters are being freed and extracted, and still finish the process within three quarters of an hour. Another advantage is the ease with which the barium is removed in order to prepare for esterification of the residues. Within a few hours the solution of unextracted amino-acids may be freed from mineral matter, and started concentrating, for a second esterification; while the process of changing masses of sodium hydroxide and potassium carbonate to chlorides and removing them by repeated concentration requires much greater expenditure of time and effort, with increased opportunities for loss of material.

The barium method has also been used with success for small amounts

of esters. In one case 92 per cent of the theoretical yield of constant-boiling valin ester was obtained from 5 grams of valin.

The casein esters were distilled, using sulphuric acid in glass-wool to absorb the gases evolved[1] instead of condensing them with liquid air. The following fractions were obtained:

	Pressure. *mm.*	Temp. Vapors. *degrees.*	Wt. of esters *grams.*
I	20	to 65	61.5
II	0.3	" 87	165.7
III	0.8	" 140	119.7
Total			346.9
Undistilled residue			63.4

Only fractions I and II were worked up for the leucin fraction.

Fraction I. The esters yielded 8.28 grams of amino-acids, which were reduced to 6.20 grams by extraction with alcohol to remove protein. Fractional crystallization yielded 2.65 grams of leucin-valin mixture. From this, 1.70 grams of lead-leucin, equivalent to 0.95 gram of leucin, was obtained.

Analysis: 0.3023 gm. substance; 0.1954 gm. $PbSO_4$; 44.14 per cent Pb.
Calculated for $Pb(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

The leucin was regenerated, and gave the following rotation in 20 per cent hydrochloric acid:

0.4851 gram of substance; 11.31 grams of total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 0.83°.

$$[\alpha]_D^{20^\circ} = + 20.3^\circ$$

Calculated from the rotation (cf. p. 400, preceding paper), the substance consisted of 21.6 per cent or 0.20 gram d-isoleucin and 0.75 gram l-leucin.

The filtrate from the lead-leucin yielded 1.63 grams of valin.

Analysis: 0.1221 gram substance; 0.2288 gram CO_2; 0.1045 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.24 per cent	51.10 per cent.
H	9.47 "	9.75 "

[1] Levene and Van Slyke: *Biochem. Zeitschr.*, x, p. 214, 1908.

The mother liquors from the above leucin-valin mixture contained mostly alanin, but yielded by crystallization from dilute alcohol 0.50 gram of valin.

Analysis: 0.0975 gram substance; 0.1829 gram CO_2; 0.0857 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	51.17 per cent.
H.....	9.47 "	9.82 "

Fraction II. The esters were saponified with about 800 cc. of water, and the solution left over night to complete crystallization. 38.11 grams of leucin (crop *a*) accompanied by a small amount of valin were obtained. The substance showed, on analysis, 54.46 per cent C, 9.80 per cent H, instead of 54.92 per cent C and 9.99 per cent H. Estimated from the analysis, the fraction should contain 33.4 grams of leucin isomers, 4.7 grams of valin. Twenty grams were submitted to the lead separation, the leucin being removed by two precipitations (cf. p. 396, preceding paper), yielding respectively 27.20 and 4.51 grams of lead-leucin, equivalent together to 17.80 grams of leucin.

Analyses: (1st ppt.) 0.3056 gram substance; 0.1973 gram $PbSO_4$; 44.10 per cent Pb.
(2d ppt.) 0.3276 gram substance; 0.2120 gram $PbSO_4$; 44.19 per cent Pb.
Calculated for $Pb(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

The leucin was regenerated from the lead salt.

Analysis: 0.1134 gram substance; 0.2279 gram CO_2; 0.1021 gram H_2O.

	Calculated for $C_6H_{13}O_2N$	Found:
C.....	54.92 per cent	54.80 per cent.
H.....	9.99 "	10.07 "

Rotation in 20 per cent hydrochloric acid: 0.6984 gram substance; 18.994 grams total solution; observed rotation + 1.32°.

$$[\alpha]_D^{20^\circ} = + 17.23^\circ$$

From the rotation:

$$\text{Isoleucin} = 100 \times \frac{17.23 - 15.6}{21.8} = 7.5 \text{ per cent; leucin} = 92.5 \text{ per cent.}$$

The filtrate from the second precipitate of lead-leucin yielded 1.77 grams of valin.

Analysis: 0.1286 gram substance; 0.2403 gram CO_2; 0.1086 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	50.94 per cent.
H.....	9.47 "	9.44 "

Rotation in 20 per cent hydrochloric acid: 0.4800 gram substance; 22.360 grams total solution; + 1.08° rotation in 1.894 dm. tube; sp. gr., 1.10.

$$[\alpha]_D^{20^\circ} = + 24.2^\circ$$

The alcoholic filtrate from the valin yielded a slight amount of brown syrup, similar in appearance to crude prolin.

The above yields indicate for the total 38.11 grams, constituting the first crop of crystals, 31.37 grams of leucin, 2.54 grams of isoleucin, 3.37 grams of valin, 0.83 gram not recovered, probably largely prolin.

A second crop (*b*) of amino-acids, weighing 40.63 grams, was obtained by concentrating the filtrate from the first *in vacuo* to a small volume, and washing the product with 60 per cent alcohol. Analysis showed 52.08 per cent C, 9.60 per cent H, indicating 9 grams of leucin in the substance (8.7 grams were found). The acids did not have the appearance of the typical leucin-valin mixture, probably because some prolin was present. Allowance was erroneously made for the possible effect of alanin on the analysis, and enough lead to precipitate 17 grams of leucin (65 cc.) used in the separation. As a result the lead precipitate contained some valin. The lead content was high and the regenerated acids, 11.45 grams, gave 54.03 to 54.01 per cent C, and 9.85 to 9.91 per cent H, indicating that they were one-fourth valin. 8.96 grams were dissolved in 60 cc. water plus 10 cc. ammonia, and precipitated with 28 cc. $\frac{M}{1}$ lead acetate, equivalent to 7.34 grams of leucin. 11.20 grams of lead leucin were obtained. The filtrate was concentrated *in vacuo* to about 30 cc. and 3 cc. concentrated ammonia added, precipitating 0.91 gram more of lead salt, making a total of 12.11 grams, equivalent to 6.80 grams of leucin.

Analyses: (1st ppt.): 0.3242 gram substance; 0.2111 gram $PbSO_4$; 44.47 per cent Pb.
(2d ppt.): 0.1966 gram substance; 0.1270 gram $PbSO_4$; 44.12 per cent Pb.
Calculated for $Pb(C_6H_{12}O_2N)_2$, 44.29 per cent Pb.

Analysis of regenerated leucin: 0.1156 gram substance; 0.2321 gram CO_2; 0.1070 gram H_2O.

	Calculated for $C_6H_{13}O_2N$:	Found:
C.....	54.92 per cent	54.76 per cent.
H.....	9.99 "	10.36 "

Rotation in 20 per cent hydrochloric acid: 0.7322 gram substance; 19.054 grams total solution; sp. gr., 1.10; rotation in 1.894 dm. tube, + 2.06°.

$$[\alpha]_D^{20^\circ} = + 25.85^\circ$$

Calculated from the rotation, the substance is 47.0 per cent isoleucin, 53.0 per cent leucin. The filtrate from the lead-leucin gave 2.00 grams of valin.

Analysis: 0.1453 gram substance; 0.2751 gram CO_2; 0.1247 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	51.63 per cent.
H.....	9.47 "	9.60 "

From the above figures, the original 11.45 grams of acids in the first lead precipitate consisted of 4.60 grams of leucin, 4.09 grams of isoleucin, 2.56 grams of valin, 0.20 gram unaccounted for.

Because of the original appearance of crop *b* in this ester fraction, the filtrate from the first lead precipitate was not concentrated to dryness, but reduced *in vacuo* only until crystallization of valin began. About an equal volume of alcohol was added, and the solution left over night in the refrigerator. The crystals were washed with 60 per cent alcohol, and 15.23 grams of valin obtained.

Analysis: (1) 0.1033 gram substance; 0.1944 gram CO_2; 0.0895 gram H_2O.
(2) 0.1523 gram substance; 0.2874 gram CO_2; 0.1316 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	(1) 51.34 per cent.
		(2) 51.47 "
H.....	9.47 "	(1) 9.68 "
		(2) 9.67 "

Rotation in 20 per cent hydrochloric acid: 0.4720 gram substance; 11.188 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.10°.

$$[\alpha]_D^{20^\circ} = + 27.4^\circ$$

To the mother liquors a small amount of alcohol-insoluble amino-acid regained from the mother liquors of main crop *b* was added, and the solution concentrated until crystallization was again well under way, then alcohol added as before. 7.78 grams more of valin was obtained.

Analysis: 0.1324 gram substance; 0.2465 gram CO_2; 0.1140 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	50.78 per cent.
H.....	9.47 "	9.63 "

The valin was once recrystallized by dissolving in hot water and adding alcohol.

Analysis: 0.1306 gram substance; 0.2449 gram CO_2; 0.1125 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	51.16 per cent.
H.....	9.47 "	9.64 "

Rotation in 20 per cent hydrochloric acid: 0.4930 gram substance; 11.403 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.04°.

$$[\alpha]_D^{20^\circ} = + 25.3^\circ$$

The amino-acids from the mother liquors were freed from prolin with alcohol, and changed to copper salts, of which 4.81 grams were obtained. Analysis indicated alanin-copper, with a small admixture of valin, but the amount of the latter was not sufficient to isolate.

The above analysis indicates, for the 40.63 grams of the second

crop (*b*) of crystals, 4.60 grams of leucin, 4.09 grams of isoleucin, 25.57 grams of valin, 6.37 grams loss and other amino-acids.

This makes the total yield of the leucin fraction:

Fraction.	Leucin grams.	Isoleucin. grams.	Valin. grams.
Ia	0.75	0.20	1.63
Ib	.....	....	0.50
IIa	31.37	2.54	3.37
IIb	4.60	4.09	25.57
Total	36.72	6.63	31.07
Per 100 grams of casein	7.92	1.43	6.69

EDESTIN.

As a combination of direct crystallization and esterification had previously been used with success in obtaining the leucin fraction,[1] particularly from relatively small amounts of protein, this method was employed for edestin. The process is more complicated, but theoretically should yield more nearly the total amount of the leucin fraction than simple esterification, because the amino-acids gained by direct crystallization escape the losses from anhydride formation, incomplete extraction, etc., that may accompany esterification. Its actual applicability, however, appears to vary with the nature of the protein, for the crystallized leucin may carry with it impurities, the removal of which results in more loss than would occur by esterification. This was our experience with casein. As a general method direct esterification, as applied in the preceding casein hydrolysis, seems preferable. In the case of edestin, the leucin and valin from the crystallized acids were not so pure as those obtained from the esters, but they appeared sufficiently so to justify an approximate estimation of their relative amounts in the protein.

Two hundred grams of edestin, calculated ash and moisture free, were hydrolyzed by 12 hours boiling with 25 per cent hydrochloric acid. The hydrochloric acid was partially removed by concentration *in vacuo*, the solution then diluted to 7 liters, and the remainder of the chlorine removed by means of precipitated

[1] Levene and Mandel: *Biochem. Zeitschr.*, v, p. 36, 1907. Levene and Van Slyke: *Ibid.*, xiii, p. 450, 1908.

lead oxide and silver sulphate.[1] The slightly yellow solution obtained was concentrated *in vacuo* until the tyrosin crystallized. 5.34 grams of pure tyrosin needles were obtained. This exceeds somewhat the amount (2.1 grams per 100 grams edestin) found by Abderhalden, and apparently constituted practically all of the tyrosin present. The filtrate gave only a slight Millon reaction, and no tyrosin needles were observed in succeeding crops of crystals.

The filtrate was concentrated *in vacuo*, and yielded three crops of crystals, weighing 16.20, 7.17, and 3.10 grams respectively. The crystals, washed thoroughly with 70 per cent alcohol, were nearly white and of fairly pure appearance, both microscopically and in mass. The first two crops were dissolved together and, as their composition was uncertain, enough lead added to precipitate 17 grams of leucin. 21.88 grams of lead-leucin, equivalent to 12.28 grams of leucin, were obtained.

Analysis: (1) 0.3169 gram substance; 0.2026 gram $PbSO_4$; 43.66 per cent Pb.
(2) 0.4689 gram substance; 0.3004 gram $PbSO_4$; 43.75 per cent Pb.
Calculated for Pb $(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

The crude valin from the filtrate was recrystallized, but could not be obtained perfectly pure. 7.05 grams of the following composition were obtained, the mother liquor being added to the main solution for esterification.

Analysis: 0.1603 gram substance; 0.3045 gram CO_2; 0.1275 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	51.81 per cent.
H.....	9.47 "	8.90 "

Rotation in 20 per cent hydrochloric acid: 0.5225 gram substance; 11.124 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.16°.

$$[\alpha]_D^{20^\circ} = + 25.9^\circ$$

The rotation agrees with those found by other authors for natural valin (cf. footnote p. 415, preceding article). The valin was transformed

[1] Levene and Van Slyke: *Biochem. Zeitschr.*, xiii, p. 448, 1908.

into copper salt, which gave the following figures on combustion: 0.1865 gram substance; 0.2825 gram CO_2; 0.1107 gram H_2O; 0.0521 gram CuO.

	Calculated for $Cu(C_5H_{10}O_2N)_2$	Found:
C.....	40.57 per cent	41.30 per cent.
H.....	6.64 "	6.83 "
Cu.....	21.50 "	22.30 "

The third crop of crystals gave 2.08 grams of lead salt, equivalent to 1.17 grams of leucin.

Analysis: 0.3161 gram substance; 0.2052 gram $PbSO_4$; 44.34 per cent Pb.
Calculated for Pb $(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

From the filtrate by recrystallization 1.02 grams of valin were obtained, of the following composition:

0.1603 gram substance; 0.2967 gram CO_2; 0.1336 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	50.47 per cent.
H.....	9.47 "	9.32 "

The mother liquors from the crystals were concentrated and submitted to two esterifications. The esters boiling below 90° at 0.5 mm. were worked up for leucin and valin. The less soluble crystal fractions, containing the leucin fraction, were combined and the leucin precipitated as usual. 4.94 grams of lead-leucin, equivalent to 2.77 grams of leucin, were obtained.

Analysis: 0.3061 gram substance; 0.1991 gram $PbSO_4$; 44.44 per cent Pb.
Calculated for Pb $(C_6H_{12}O_2N)_2$: 44.29 per cent Pb.

The filtrate gave 3.08 grams of valin.

Analysis: 0.1350 gram substance; 0.2531 gram CO_2; 0.1131 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C.....	51.24 per cent	51.14 per cent.
H.....	9.47 "	9.34 "

Rotation in 20 per cent hydrochloric acid: 0.5102 gram substance; 11.275 grams total solution; sp. gr., 1.10; rotation in 0.865 dm. tube, + 1.10°.

$$[\alpha]_D^{20^\circ} = + 25.6^\circ$$

Because the leucin from direct crystallization, obviously from the low lead content (43.7 instead of 44.3 per cent), contained slight amounts of impurities, which might affect the rotation somewhat, the polarimetric determination of the proportion of isoleucin was not applied. The free leucin from all the lead precipitates gave on analysis 54.98 per cent C, 9.30 per cent H, and showed a specific rotation of + 19.7° in 20 per cent hydrochloric acid.

The results are summarized as follows:

	Leucin. *grams.*	Valin. *grams.*
Direct crystallization	13.45	8.07
Esters	2.77	3.08
Total	16.22	11.15
Per 100 grams of edestin	8.1	5.6

Although the figures are minimal, and doubtless considerably short of the true values, the relative proportions of leucin and valin are, we believe, approximately correct.

A comparison of our figures for the leucin fractions of casein and edestin with the figures of Abderhalden is given below. They represent grams of amino-acid per 100 grams of protein.

	CASEIN.		EDESTIN.	
	Levene and Van Slyke	Abderhalden.[1]	Levene and Van Slyke	Abderhalden.[2]
Leucin	7.92	10.5	8.1	20.9
Isoleucin	1.43			
Valin	6.69	1.0	5.6	0.45
Total leucin fraction	16.04	11.5	13.7	20.9

The most striking differences between our results and Abderhalden's are in the valin, which apparently was nearly all calculated in with the leucin by Abderhalden (cf. footnote, p. 393, preceding article). The probability that much more valin was present

[1] *Zeitschr. f. physiol. Chem.*, xlvi, p. 23.

[2] *Ibid.*, xxxvii, p. 499 (no valin reported); xl, p. 249 (slight amount of valin reported from leucin fraction of previous hydrolysis).

than could be isolated was recognized by Abderhalden.[1] That our total figures for leucin plus valin in edestin are much lower, is probably partly due to the fact that Abderhalden's figures must have been based on crude products, as shown by the fact that but a small fraction of the valin present was separated from the leucin. Our figures, although we do not believe they are as high as might be obtained, are based upon approximately pure substances. Abderhalden's casein figures were doubtless also based on incompletely purified products, but in this case our total yield is 40 per cent larger, although based entirely on analytically pure substances. The difference is probaby due to a more complete extraction of the esters freed by the barium method, as our yield was exceptionally high, 347 grams of esters from 464 grams of casein.[2]

Considering the lack of even approximate accuracy in Abderhalden's figures for the leucin fraction, which are taken from what may be considered standard hydrolyses by the methods previously available, it appears probable that the figures for the leucin fraction in most of the protein hydrolyses hitherto published are in need of revision.

[1] "Bis jetzt ist unter den Spaltungsprodukten der Proteine nur d-Valin aufgefunden worden, und zwar meist nur in geringer Ausbeute. Es unterliegt jedoch keinern Zweifel, dass das Valin in Wirklichkeit in grösseren Mengen vorhanden ist. Es zeigt die Neigung, mit -lLeucin und d-Isoleucin Mischcrystalle zu bilden und ist von diesern nur unter grossen Verlust in völlig reinem Zustande zu trennen." E. Abderhalden, *Neuere Ergebnisse der Esweisschemie*, p. 38, 1909.

[2] The Dennstedt method was used for the numerous combustions connected with the foregoing work, and was found exceedingly satisfactory.

THE NATURE OF THE ACID SOLUBLE PHOSPHORUS COMPOUNDS OF SOME IMPORTANT FEEDING MATERIALS.[1]

By E. B. HART and W. E. TOTTINGHAM.

(*From the Agricultural Chemical Laboratory of the University of Wisconsin.*)

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In 1900 Posternak published his first paper[2] on the occurrence of phytin in vegetable tissue. This was followed by subsequent papers[3] in which he described the separation of this body from the seeds of the red fir, pumpkin, pea, bean, white and yellow lupine and the potatoe. In 1904 Patten and Hart[4] isolated phytic acid from wheat bran, and in 1907 Suzuki[5] succeeded in separating the same body from rice bran.

The indications are that phytic acid as a complex salt of potassium, magnesium and calcium, is widely distributed in nature, particularly in the seeds as a reserve material. In our early work[6] on this subject it was found that the natural grains, such as corn, oats and wheat, yielded to an extraction with dilute hydrochloric acid, from 30 to 50 per cent of their total phosphorus. This, interpreted in the light of available methods, meant that practically the entire hydrochloric acid-soluble phosphorus was organic in form.

The investigation incorporated in this paper is confined to a continuation of our study on the nature of the phosphorus-bearing bodies contained in this dilute hydrochloric acid extract of a few of our natural feeding materials.

Representatives of different botanical families have been chosen. Although the work of Palladin[7] and Posternak had

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] *Rev. gén. de botanique*, xii, pp. 5 and 65, 1900.

[3] *Compt. rend. de l'acad. des sci.*, no. 3, no. 5, no. 8, cxxxvii, 1903.

[4] *Amer. Chem. Journ.*, xxxi, no. 5.

[5] Bulletin of College of Agriculture, Tokio Imperial University, vii, no. 4.

[6] Hart and Andrews: *Amer. Chem. Journ.*, xxx, no. 6.

[7] *Zeitschr. f. Biol.*, 1894, p. 199.

already revealed the presence of phytin in the potatoe, a representative of the *Solanaceæ;* in the seeds of the pea and bean—*Leguminosæ*, and in the black mustard seeds—*Cruciferæ*, nevertheless it was thought not impossible that a chemical difference might obtain in the phosphorus complexes in other species of these families of plants. In addition, studies of parts of the plant other than the seeds were undertaken. With this end in view, representatives of the *Gramineæ*, *Cruciferæ* and *Leguminosæ* were chosen.

In our choice of materials we have always selected those parts of the plant that are used preëminently for direct animal feeding. It is not to be doubted that the different parts of a plant will vary, not only in the proportion but also in the nature of its phosphorus bearing bodies. We would expect to find bodies of greater mobility and simpler structure in the stems of plants than in the seeds. This is true of nitrogenous structures, as exemplified in our hays, where the simple amino acids predominate, as compared with a much lower content of these forms in the seeds. For example, in our previous work[1] it was found that while 70 per cent of the phosphorus in alfalfa hay was soluble in dilute hydrochloric acid, 52 per cent of this acid soluble phosphorus was inorganic in form. This is very different from the condition prevailing in some of our seeds, as oats, corn and wheat, where 30 to 50 per cent of the total phosphorus was acid soluble, but practically all in organic form.

Isolation of Phytic Acid from the Corn Grain (Zea mays).

Ten kilos of finely ground corn meal were extracted with 0.2 per cent hydrochloric acid, strained through cheesecloth and filtered through paper. The clear brown solution was treated with copper acetate in dilute acetic acid solution to facilitate the removal of any bases existing in combination with phytic acid. After thoroughly washing, the copper precipitate was suspended in water and decomposed by hydrogen sulphide. The filtrate from the copper sulphide was made alkaline with sodium hydroxide and precipitated with barium chloride. The barium salt was washed free from alkali, suspended in water and dilute sul-

[1] Hart and Andrews: *loc. cit.*

phuric acid added in sufficient quantity to decompose the salt and throw down the barium as a sulphate. After removal of the barium sulphate by filtration, the filtrate was again precipitated in alkaline solution with barium chloride and treated as before. This process was repeated three or four times and after final removal of the barium, copper acetate was added in excess. The copper precipitate was filtered, thoroughly washed with water, and finally suspended in water and decomposed by hydrogen sulphide. The copper sulphide was removed by filtration and the filtrate evaporated on the water bath to a syrupy consistency.

The residue was dark in color and sharply acid to indicators, Analysis of the acid dried at 100° C. gave the following results:

0.113 gram of substance gave 0.0426 gram CO_2 = 10.3 per cent C.
0.200 gram substance gave 0.187 gram $Mg_2P_2O_7$ = 26.08 per cent P.

	Calculated for $C_2H_8P_2O_9$:	Found:
C	10.08 per cent	10.30 per cent
P	26.07 "	26.08 "

Decomposition of the Compound into Inosite and Phosphoric Acid. In this investigation an unweighed portion of the acid was heated in a sealed tube with 50 cc. of 30 per cent sulphuric acid at a temperature of 155 to 160° C. for five hours. After cooling, the tube was opened, the sulphuric and phosphoric acids removed by barium hydroxide and the excess of barium with carbon dioxide. The filtrate was evaporated nearly to dryness, taken up with hot water and filtered from the remaining barium carbonate. This filtrate was treated with absolute alcohol and ether until a cloudiness was produced. It was then surrounded by ice and allowed to stand. A crystalline precipitate separated which after recrystallization was identified as inosite. It gave the reactions of Scherer and Gallois and melted at 220° C. (uncorrected). Inosite melts at 218° C. (uncorrected). The substance dried at 110° C. gave the following results:

0.100 gram of substance gave 0.148 gram CO_2 and 0.062 gram H_2O.

	Calculated for $C_6H_{12}O_6$:	Found:
C	40.00 per cent.	40.36 per cent.
H	6.66 "	6.88 "

The isolation of inosite and the data on the ultimate analysis identify the body separated from corn as phytic acid.

Preparation of Phytin.

Two kilos of corn meal were extracted with water for several hours with frequent agitation. The extract was strained through cloth and finally filtered through paper to a clear solution. The filtrate was treated with a large volume of 95 per cent alcohol, which threw down a voluminous flocculent precipitate. The precipitate was allowed to settle to the bottom, the supernatant liquid siphoned off and the precipitate washed with alcohol by decantation. It was then redissolved in a small volume of water, filtered from an insoluble residue, reprecipitated with alcohol and washed as before by decantation. The precipitate was finally brought upon the filter, washed with alcohol and ether and dried at 100° C. The product was a yellow amorphous powder, very rich in phosphorus, and but slightly soluble in water. Apparently some substance, very probably of a protein nature—as the substance still gave color reactions for protein—was contaminating the material and modifying the solubilities of the entire mass. The material manifestly does not represent a pure product. Nevertheless a partial analysis is appended below, which indicates that we had an impure salt of phytic acid and that the same bases that were found in a preparation of phytin from wheat bran[1] were to be found here.

	Per Cent
Ca	0.48
K	5.60
Mg	6.70
P	14.17

Distribution of Phytin in the Seed.

In the wheat kernel phytic acid as a salt exists largely in the outer aleurone layers and consequently is found in very large proportion in wheat bran. In order to determine whether similar distribution obtained in the corn kernel this seed was mechanically divided into three parts—the outer layer or corn bran

[1] Patten and Hart: *Amer. Chem. Journ.* xxxi, no. 5, 1904.

(pericarp), the germ and the starch and gluten cells (endosperm). The following table shows the distribution of total and dilute acid soluble phosphorus. These determinations were made on the air-dried material.

	Total P. Per Cent	P Soluble in 0.2 Per Cent HCl, Per Cent
Entire seed	0.29	0.13
Corn bran	0.13	0.00
Corn germ	0.38	0.13
Corn gluten	0.42	0.15

It is clear from these data that in the maize grain, phytin is not localized in the outer layers. It is probably very nearly accurate to consider that the phosphorus of cereal grains soluble in dilute hydrochloric acid largely exists as phytin. In this instance there appears to be none in the outer skin or seed coats, while there is, on the contrary, more or less uniform distribution throughout the entire seed.

Isolation of Phytic Acid from the Oat Kernel (Avena sativa).

Eight to ten kilos of finely ground seed were extracted with 0.2 per cent hydrochloric acid, strained through cheese cloth and filtered through paper. The details of the preparation and purification of the acid were in every particular similar to those given under the separation of this product from corn, and consequently it will be unnecessary to repeat them.

The final preparation was black in color, of a syrupy consistency and a sharp acid taste. It was dried at 100° C. and gave the following results on analysis:

0.128 gram of substance gave 0.048 gram of CO_2 = 10.22 per cent C.
0.291 gram of substance gave 0.271 gram of $Mg_2P_2O_7$ = 25.97 per cent P.

	Calculated for $C_6H_8P_2O_9$:	Found:
C	10.08 per cent.	10.22 per cent.
P	26.07 "	25.97 "

Decomposition into Inosite and Phosphoric Acid.

An unweighed portion of the acid was treated in a sealed tube with 30 per cent sulphuric acid at 155 to 160° C. for five hours.

After removing the sulphuric and phosphoric acids, the residual solution was evaporated nearly to dryness, treated with alcohol, and ether, iced and allowed to stand. The crystalline precipitate which separated was again recrystallized, washed with ether and dried at 100° C.

This body melted at 217.5° C. (uncorrected) and gave the reactions of Scherer and Gallois. Inosite melts at 218° C. (uncorrected). These reactions and the melting point fully identified the body as inosite and established the presence of the phytic acid radical in the oat grain.

Preparation of Phytin from Oats.

Two to three kilos of the finely ground grain were extracted with water and the extract finally obtained clear by frequent filtration through paper. The further separation and purification of the preparation was conducted in exactly the same manner as has been previously detailed in the case of the preparation from the corn grain. The color reactions for protein persisted even after a third resolution and reprecipitation of the substance. The final preparation was a white friable powder and but partially soluble in water. Estimation of the bases and phosphorus gave the following figures:

	Per Cent
Ca	8.6
Mg	4.7
K	1.5
P	16.7

Distribution of Phytin in the Seed.

The seed was mechanically separated into the hull or bran layers (pericarp) and kernel. The former is fibrous and forms a considerable portion of the grain. The latter consists of the aleurone layer and starch cells (endosperm) and the embryo. No further divisions were made. The analyses are reported on the air-dried material.

	Total P. Per Cent	P Soluble in 0,2 Per Cent HCl. Per Cent
Entire seed	0.41	0.18
Oat kernel	0.41	0.22
Oat hull	0.41	0.09

It is apparent from the data that while the seed coats carry a total amount of phosphorus comparable with the other parts of the grain, the proportion of phytin in the outer layers is relatively small. On the other hand it constitutes 50 per cent of the total phosphorus bearing bodies in the remaining parts of the seed.

Isolation of Phytic Acid from Barley (Hordeum sativum).

Five kilos of finely ground barley were extracted with 0.2 per cent hydrochloric acid. The extract was filtered clear and precipitated with copper acetate. This precipitate was decomposed with hydrogen sulphide, the copper sulphide removed and further manipulation continued as has already been described. The product was a dark sticky mass and less fluid when perfectly dry than the preparations so far studied from the other grains. It was dried at 110° C. and gave the following results:

0.154 gram of substance gave 0.058 gram of CO_2 = 10.5 per cent C.
0.485 gram of substance gave 0.450 gram of $Mg_2P_2O_7$ = 25.88 per cent P.

	Calculated for $C_2H_8P_2O_9$:	Found:
C	10.08 per cent	10.54 per cent
P	26.07 "	25.88 "

Decomposition of the Product.

An unweighed portion was decomposed under pressure with 30 per cent sulphuric acid heated to 155° C. for five hours. After removing the sulphuric and phosphoric acids, the clear solution was evaporated to 25 cc., cooled and treated with alcohol and ether. After icing and allowing to stand a few hours, a crystalline precipitate separated which after recrystallization was dried at 110° C. This substance had a melting point of 219° (uncorrected). It gave the Scherer reaction and a carbon determination gave the following results:

0.120 gram of substance gave 0.174 gram of CO_2 = 39.5 per cent C.

	Calculated for $C_6H_{12}O_6$:	Found:
C	40.0 per cent	39.5 per cent

These results fully identified the body as inosite and furnished conclusive evidence that the preparation from barley was phytic acid.

Preparation of Phytin.

Five kilos of ground barley were extracted with water for 36 hours under frequent stirring and in the presence of chloroform and toluol to inhibit bacterial fermentations. The liquid, both supernatant and expressed, was filtered through paper, boiled to coagulate the proteins, filtered, concentrated and precipitated with 95 per cent alcohol. This precipitate was filtered off, digested with water and again filtered from an insoluble residue. The clear solution was treated with alcohol and the precipitate filtered out. This was redigested with water and the process as above described repeated a third time. The preparation was difficultly but completely soluble in water and after drying at 100° C. for five hours showed the following proportion of bases and phosphorus:

	Per Cent
Ca	0.00
Mg	7.9
K	11.2
P	14.46

No calcium was present in this preparation from barley.

Distribution of Phytin in the Seed.

The separation of the entire seed into the outer fibrous coats (pericarp), here designated as bran, and into the aleurone layer, starch cells and embryo (kernel) was carried out mechanically. The separate portions of the seeds gave the following results. The determinations were made on air-dried materials:

	Total P. Per Cent	P Soluble in 0.2 Per Cent HCl. Per Cent
Entire seed	0.50	0.19
Barley bran	0.22	0.15
Barley kernel	0.57	0.17

These results make it manifest that phytin is not concentrated in the outer seed coats, although it does constitute a very large

proportion of the total phosphorus existing there. Phytin appears to be distributed throughout the entire seed.

Study of the Dilute Hydrochloric Acid Soluble Phosphorus in Rutabagas (Brassica rutabaga).

Posternak has already isolated phytic acid from the potatoe. The fact that roots and tubers are agriculturally valuable because of the store of nutrients which they deposit in their subterranean branches or roots lead us to select for investigation another representative of this class of materials.

Twenty-five to thirty kilos of rutabagas were thinly sliced and dried. After grinding, the material was extracted with 0.2 per cent hydrochloric acid and the clear extract filtered and precipitated with copper acetate. The further treatment and manipulation was exactly comparable to that followed in the preparation of phytic acid from the grains. The product finally obtained, dried down to a gummy, dark-colored mass. It gave no protein reactions and was free from nitrogen, but had partly lost its original solubility in water. It reduced Fehling's solution, an important distinction from phytic acid, which does not have this reducing property. An ultimate analysis of this preparation, dried at 100° C., gave the following results:

	Per Cent
P	16.92
C	17.81
H	3.74

This first preparation was again treated with water, filtered from an insoluble residue, and the insoluble barium salt again prepared in alkaline solution. After its resolution in dilute sulphuric acid and a repeated precipitation and solution a second and third time, the material was separated as a copper salt, decomposed with hydrogen sulphide, evaporated and dried at 100° C. A phosphorus determination on this preparation showed 25.98 per cent. Too small an amount, however, of the second preparation was available for further analytical work. It did not directly reduce Fehling's solution, indicating that the further process of purification had removed a contaminating and reducing substance from preparation I.

Hydrolysis of Preparation I.

An unweighed quantity of preparation I was heated for five hours with 30 per cent sulphuric acid in a sealed tube at 155° to 160° C. After removing the sulphuric and phosphoric acids with barium hydroxide the solution was concentrated and treated with 95 per cent alcohol and ether. The mixture was iced and allowed to stand several days. A trace of a white amorphous substance separated which was finally collected on a filter for further examination. It did not give the reactions of Scherer and Gallois. It reduced Fehling's solution in contradistinction to the behavior of inosite with this solution. These tests exclude the possibility of this body being inosite. Further, it makes it very improbable that phytic acid exists in rutabagas.

A quantitative extraction of the dried rutabagas with dilute 0.2 per cent hydrochloric acid showed that 88 per cent of the total phosphorus was soluble in this menstruum. Application of the Hart-Andrews method[1] for inorganic phosphorus to the hydrochloric acid extract, showed that 73 per cent of the acid soluble phosphorus was inorganic in form. However, in this estimation on rutabagas somewhat more nitric acid than the method specified was used to effect precipitation by the neutral molybdate. The rapidity with which the separation of the molybdate took place when just sufficient nitric acid was present indicates the suppression of some retarding influence on the molybdate separation, rather than a progressive hydrolysis of organic forms of phosphorus. It appears from the data that there was no phytin in the rutabagas examined and that the greater part of the hydrochloric acid-soluble phosphorus exists in inorganic forms. It is, however, probable that in addition to the inorganic forms, there was in small amounts some complex of phosphoric acid paired with an unknown organic radical. The amount of material obtained was too small for a thorough study of these preparations and this work is only preliminary to their further investigation.

[1] *Amer. Chem. Journ.*, xxx, no. 6, December, 1903.

Examination of the Dilute Hydrochloric Acid Extract of Cured Alfalfa Hay (Medicago sativa).

The material used was clean, field-cured alfalfa, cut in early bloom. Six to eight kilos of the air-dried material were finely chopped, ground and extracted with 0.2 per cent hydrochloric acid. The details for the separation of phytic acid were strictly followed. The final preparation dried at 100° C. was a dark, limpid liquid, differing in this physical respect from any of the grain or rutabaga preparations, these usually being either thick and syrupy in character, or occasionally even gummy. It gave none of the protein color reactions and was free from nitrogen. It strongly reduced Fehling's solution. An ultimate analysis gave the following results:

	Per Cent
P	16.91
C	9.19
H	3.50

This analysis differs from that of a pure phytic acid, the per cent of phosphorus being materially lower.

Hydrolysis with Strong Acids.

A portion of the preparation was hydrolyzed with 30 per cent sulphuric acid in a sealed tube at 155° C. for five hours. After removal of all the sulphuric and phosphoric acids with barium hydroxide and the excess of barium as a carbonate, the clear solution was evaporated to a small bulk; it was then treated with alcohol and ether, iced and allowed to stand several days, as in the usual procedure for the separation of inosite. Finally a small amount of an amorphous material separated. This substance was collected and tested for inosite. It gave none of the characteristic color reactions for that body and reduced Fehling's solution. These facts exclude the possibility of the existence of phytic acid in alfalfa at that stage of its development.

Hydrolysis with Dilute Acids.

2.25 grams of the material were treated with 100 cc. of a 2.5 per cent solution of sulphuric acid for two hours at the tempera-

ture of the water bath and under a reflux condenser. After removing th sulphuric and phosphoric acids a quantitative estimation of the reducing power of the filtrate was made. This was equivalent to 0.035 gram calculated as dextrose. The material in 50 cc. rotated + 1.0° in a 400 mm. tube, Soleil-Ventzke instrument, 0.035 gram of dextrose under similar conditions, should have given a rotation of approximately + .7°. As the material on hand was too small for further study, it is at present impossible to definitely decide whether this simple hexose was present. The readings are within experimental error for small amounts of dextrose.

Distillation of a small sample of the original material with strong hydrochloric acid gave no positive test for furfurol, excluding the pentose sugars in this instance as possible paired bodies with phosphoric acid.

Studies on this subject are being continued. There is strong evidence for the supposition that a class of organic phosphorus bodies, which are not phytins, exists in the transition stages of growth of the alfalfa plant.

It is, however, necessary to observe that a large part of the dilute soluble phosphorus in the material at this stage of growth was inorganic in form. Eighty per cent of the total phosphorus was soluble in 0.2 per cent hydrochloric acid, while by the Hart-Andrews method, 79 per cent of this soluble phosphorus was inorganic in form.

In the following table is displayed a summary of the analytical data covering the estimations on the several materials of total dilute acid soluble and inorganic phosphorus. The determinations were made on air-dried materials:

	TOTAL P.	P SOLUBLE IN 0.2 PER CENT HCl.	INORGANIC P.	PHYTIN P. PER CENT OF TOTAL.	INORGANIC PER CENT OF TOTAL.
	per cent.	*per cent.*	*per cent.*		
Corn grain.....	0.29	0.13	0.00	45	00
Oat "	0.46	0.22	0.00	48	00
Barley "	0.50	0.19	0.00	38	00
Rutabaga.....	0.50	0.44	0.32	00	64
Alfalfa hay.....	0.30	0.24	0.19	00	63

If we assume that the phosphorus in the dilute acid extract of the grains is practically all as phytin, and this assumption is probably very near the truth, then the amount of this form, as shown in the table under column four, can be approximately derived. Column five shows that over 60 per cent of the total phosphorus in the rutabaga and alfalfa hay was inorganic, while of that soluble in dilute hydrochloric acid the amount of inorganic phosphorus was 79 and 73 per cent respectively.

SUMMARY.

(1) Phytin is present in the seeds of the corn, oat and barley plants. It has already been isolated from the wheat kernel.[1]

(2) Phytin could not be isolated from rutabagas or alfalfa hay.

(3) It appears to be distributed throughout the entire seed of the cereal grains reported in this investigation, differing in this respect from wheat, in which it is largely localized in the outer layers.

(4) The proportion of phytin phosphorus as approximately estimated was 38 to 48 per cent of the total phosphorus in these seeds.

(5) The principal form of phosphorus in the rutabaga was inorganic, 64 per cent of the total existed as such; there was in addition some unknown organic phosphorus complex, soluble in dilute acids, nitrogen free and yielding reducing substances on hydrolysis, the phosphorus of which approximated 24 per cent of the total phosphorus of this root.

(6) In alfalfa, cut when just coming into bloom, and representing that stage of growth recognized as most suitable for forage, the inorganic phosphorus found was about 63 per cent of the total phosphorus.

(7) There was present in the dilute acid extract of alfalfa hay an organic phosphorus complex, separated by the modified Posternak method, which was nitrogen free, yielded reducing bodies on hydrolysis with acids, but did not produce inosite; it consequently was not phytic acid. The phosphorus of this body constituted about 17 per cent of the total phosphorus of the plant.

[1] Patten and Hart: *Amer. Chem. Journ.*, xxxi, no. 5.

(8) It is possible that the reducing substance, separated by dilute acids from the alfalfa hay preparation, was dextrose. Further work is necessary to fully decide this point. No pentose sugar could be separated from the same preparation.

A VOLUMETRIC METHOD FOR THE ESTIMATION OF CASEIN IN COW'S MILK.[1]

By E. B. HART.

(*From the Department of Agricultural Chemistry of the University of Wisconsin.*)

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The desirability of a rapid and accurate volumetric method for the estimation of casein in cow's milk has been felt by those engaged in milk studies or conducting food laboratories. The long official method involving the Kjeldahl process often stands in the way of a complete milk analysis, which should include the estimation of this important constituent. The method reported here was evolved three years ago, but for the reason that the much simpler, centrifugal method[2] was subsequently developed, it has never been published. The expense of the apparatus necessary for the centrifugal process may deter many laboratories engaged in dairy studies from installing such apparatus. For that reason it appears desirable to publish this volumetric method as developed at that time.

Robertson[3] in 1906 outlined the principles involved in such a method, but did not furnish the necessary constants for a direct application to cow's milk.

Early attempts to titrate milk directly with various indicators for the purpose of a casein determination have proven fruitless. No very definite and sharp end reaction could be obtained, and the disturbing influence of a varying ash content has always worked against the direct application of a volumetric method for this determination. In the method detailed below the casein

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] Twenty-fourth Annual Report of the Agricultural Experiment Station, University of Wisconsin.

[3] This *Journal*, ii, p. 317.

itself is first separated and then volumetrically estimated. This affords sharp titrations.

Previous studies upon milk casein by Söldner,[1] Courant,[2] Van Slyke and Hart,[3] have led to the theory that this body probably exists in cow's milk as a calcium casein compound, loosely joined to calcium phosphate, as suggested by Lehmann and Hempel.[4] Treatment with acid results in a displacement of the calcium and formation of the base-free protein, after which it either adsorbs or enters into loose combinations with the excess of acid and the salts formed by the added acid. In the light of the later researches of Sammis[5] and Sammis and Hart[6] there is a strong probability that this theory is inadequate as a complete explanation of the phenomenon of milk curdling by acids. However, for the purposes of this paper, further discussion of the theories of acid action on milk is irrelevant.

Numerous preparations of casein from cow's milk by the use of acids, following Hammarsten's method, have yielded a body, after removal of the precipitating acid by long washing, with acid properties and capable of liberating carbon dioxide from calcium carbonate and passing into an opalescent solution. Treatment with lime water or a fixed alkali also readily dissolves this protein to a slightly milky solution. When these alkalies were used for solution, with phenolphthalein as an indicator, titration of the excess of alkali with an acid showed that after the disappearance of color there was still alkali to be accounted for. In other words, such preparations of casein could neutralize a certain amount of alkali as indicated by phenolphthalein. It was upon this theory of action that a quantitative method for the estimation of casein was approached. It remained to be determined whether the amount of casein used and alkali neutralized bore any definite relation to each other.

[1] *Landw. Versuchsstat*, xxxv, p. 351, 1888.
[2] *Arch. f. d. ges. Physiol.*, l, p. 109, 1891.
[3] *Amer. Chem. Journ.*, xxxiii, no. 5, 1905.
[4] *Arch. f. d. ges. Physiol.*, lvi, p. 558, 1894.
[5] Twenty-fourth Annual Report of the Wisconsin Agricultural Experiment Station, p. 171.
[6] This *Journal*, vi, no. 2, 1909.

Determination of the Alkali Constant.

For the determination of the alkali constant under uniform conditions, preparations of casein were made by Hammarsten's method from mixed milk and milk from typical breeds. It was thought necessary to extend the source of casein to several breeds, in order to determine whether variations in its power to neutralize alkali obtained in preparations from such milks. Preparations were made from the mixed milk of the University herd comprising our representative breeds, and separately from the milk of pure-bred Jerseys, Guernseys, Holsteins and Ayrshires, included in the herd.

Preparation of casein. Night's milk was run through a hand separator to remove the fat and then allowed to stand cold until the next morning. One liter of this skimmed milk was diluted with eight liters of water and precipitated with dilute acetic acid. It was allowed to settle, washed several times by decantation and redissolved in dilute potassium hydroxide. It was then precipitated with acetic acid, washed by decantation and finally thrown on filter papers and washed thoroughly with water. It was next ground under 95 per cent alcohol, filtered and washed with ether and dried in an air oven at 110° C. In all cases a friable white powder free from acid was obtained.

Nitrogen and ash determinations showed that these preparations were quite uniform in regard to the amounts of these constituents.

	Nitrogen Per cent.	*Ash Per cent.*
Mixed Casein	15.57	0.66
Jersey "	15.56	0.76
Guernsey "	15.57	0.70
Holstein "	15.65	0.55
Ayrshire "	15.49	0.57

Varying amounts of the above casein preparations were weighed out in 200 cc. Erlenmeyer flasks, 75 cc. of carbon dioxide-free water added and 10 cc. of $\frac{N}{10}$ potassium hydroxide. The flasks were then stoppered and shaken until solution. After solution, which means a slight opalescence but complete disappearance of all solid particles, phenolphthalein was added and the solution titrated with $\frac{N}{10}$ sulphuric acid until disappearance of color. The data secured in this work are appended in the following table:

Table Giving the Casein Equivalent of 0.1 cc. $\frac{N}{10}$ KOH.

CASEIN.	AMOUNT USED.	$\frac{N}{10}$ H_2SO_4.	CASEIN EQUIVALENT OF 0.1 CC. $\frac{N}{10}$ KOH.
	grams.	*cc.*	
Mixed	0.1358	8.70	0.0104
"	0.2092	8.05	0.0107
"	0.2798	7.50	0.0111
"	0.3507	6.90	0.0113
Jersey	0.1149	8.90	0.0104
"	0.1994	8.15	0.0102
"	0.2103	8.05	0.0107
"	0.2464	7.70	0.0107
Guernsey	0.1438	8.65	0.0106
"	0.3032	7.25	0.0110
"	0.3118	7.15	0.0109
"	0.4310	6.15	0.0111
Holstein	0.1246	8.80	0.0104
"	0.1836	8.30	0.0108
"	0.2157	8.00	0.0107
"	0.2939	7.35	0.0110
Ayrshire	0.1718	8.40	0.0107
"	0.2016	8.10	0.0106
"	0.2465	7.75	0.0109
"	0.3532	6.80	0.0110
Average			0.0108

This table shows a marked constancy in the casein equivalent of 0.1 cc. of $\frac{N}{10}$ potassium hydroxide. The average of all the results is 0.0108. It is also apparent from a consideration of the data that the breeds as varying sources for the casein had no influence upon this constant. Purposely the variations in the amounts of casein taken cover the amounts usually found in cow's milk, and while one can see a slight increase in the value of this constant for increasing amounts of casein, its increase is too slight to introduce any appreciable error in normal milks.

These determinations were all carried on at room temperature, 18 to 22° C., and the time of solution was not over one hour. These are important considerations to keep in mind in view of the fact that the age and temperature of solutions of alkali caseinates

are factors influencing in a greater or less degree the point at which the phenolphthalein color disappears.[1]

By establishing the casein value for $\frac{N}{10}$ potassium hydroxide as 0.0108 gram per 0.1 cc., it simply requires that 10.8 grams of milk be taken for the casein estimation, in order that the per cents of casein may be read directly on the burette. The average specific gravity of normal cow's milk lies between 1.030 and 1.034. Consequently 10.5 cc. of milk would be equivalent to 10.8 grams. This is the volume that has been adopted in this method.

THE METHOD.

10.5 cc. of milk are placed in a 200 cc. Erlenmeyer flask, 75 cc. of distilled water, at room temperature, are added and 1–1.5 cc. of a 10 per cent solution of acetic acid. The flask is given a vigorous rotary motion. Usually 1.5 cc. of acetic acid gives a clear and fast filtering separation, but, occasionally, with milks low in casein, a better separation is effected if a little less acetic acid is used. The separated precipitate is now filtered through a 9–11 cm. filter. As the casein accumulates on the filter, there is a marked retardation of the filtering process. This can again be made rapid by conducting a fine stream of cold water against the upper point of contact of filter paper and casein. This loosens the casein mass and accumulates it at the apex of the filter. This is all essential to the proper working of the process.

It is absolutely necessary that all loosely combined or adsorbed acetic acid be removed from the precipitate by a thorough washing and also that cold water be used in the process, in order to maintain the casein in a loose, easily soluble mass. The readiness with which the casein dissolves in the dilute alkali is largely determined by the temperature to which it has been subjected in the manipulations. A temperature not exceeding 20° C., and one, that in our experience prevented the formation of large compact masses, is recommended. The particles of casein adhering to the glass of the precipitating flask need not be removed as the bulk of the precipitate is again to be returned to the same flask. They should, however, be thoroughly washed. The washings should continue until at least 250 to 300 cc. of filtrate

[1] Sammis and Hart: This *Journal*, vi, no. 2, 1909.

have accumulated. This insures removal of all acid. If the process has been properly conducted and the right amount of acid used in the precipitation, the filtrate will be perfectly clear.

The precipitate, together with the filter paper, is now returned to the flask in which the precipitation was made, 75 to 80 cc. of neutral carbon dioxide free water added and then a few drops of phenolphthalein and 10 cc. of $\frac{N}{10}$ potassium hydroxide. The flask is stoppered with a rubber stopper, and vigorously shaken, either by hand or in a machine, until solution. Complete solution is easily indicated even in the presence of the filter paper by the disappearance of the white casein particles, that otherwise would settle to the bottom. After solution, the stopper is rinsed off with neutral, carbon dioxide free water and immediately titrated with $\frac{N}{10}$ acid to the disappearance of the red color. It is imperative that a blank should be run parallel with the entire determination. In our experience it was always found that even when all ordinary precautions were taken to secure neutrality, a blank of 0.2-0.3 cc. acidity was obtained. The correction for the blank—and in our laboratory it was invariably in the same direction—is made by its addition to the number of cc. of acid used in the titration. The difference between this corrected acid reading and the 10 cc. of alkali used gives directly the per cent of casein in the milk.

EXAMPLE: Suppose it took 6.7 cc. of $\frac{N}{10}$ sulphuric acid in the titration, and the blank was 0.2 cc., then the per cent of casein becomes 10–6.9 or 3.1 per cent. The acid burette used was graduated in twentieths, and titrations were possible to that degree of fineness. The results secured by this method are shown in the following table, where determinations on milks from a variety of breeds are compared with the method adopted by the Official Agricultural Chemists. The determinations given in the third column were kindly made by Professor Sammis of the Dairy Department of the University, and show a close agreement with my own figures. In all Kjeldahl determinations the factor 6.38 was used.

Comparison of Results by Official and Volumetric Methods.

	OFFICIAL METHOD	VOLUMETRIC METHOD (HART).	VOLUMETRIC METHOD (SAMMIS).
	per cent.	*per cent.*	*per cent.*
Jersey	3.78	3.75	
"	3.12	3.05	
"	3.64	3.70	3.70
"	2.87	2.85	2.85
Guernsey	3.22	3.20	
"	3.10	2.90	
"	2.71	2.75	2.72
"	2.64	2.60	2.45
Holstein	2.13	2.10	
"	1.90	1.85	
"	2.36	2.35	2.25
Ayrshire	3.54	3.45	
"	2.30	2.25	
"	3.39	3.25	
Brown Swiss	2.73	2.60	
" "	2.37	2.30	2.30
" "	2.84	2.80	

The method is rapid and, if properly conducted, can give results in less than two hours.

ON PREFORMED HYPOXANTHIN.

By V. N. LEONARD and WALTER JONES.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, July 22, 1909.)

The idea that endogenous uric acid has its origin in the leucocytes has been found in accordance with an accumulation of evidence so uniform in its indications that what was originally a speculation has of late come to be regarded almost as a logical necessity. In the first place Kossel's discovery of purin derivatives as hydrolytic products of nucleic acids showed the presence in the nuclei of a substance which could furnish the fundamental chemical grouping sufficient for the production of uric acid. Later, the entire mechanism of a process at action in the tissues was shown by which either nucleic acid or its purin decomposition products may give rise to uric acid. First a ferment (nuclease) was found which splits off from nucleic acid two amido-purins, guanin and adenin: then a ferment was shown present in certain tissues under whose influence oxy-purins are oxidized to uric acid: and finally the discovery of two independent disamidizing ferments capable of changing the amido-purins into corresponding oxy-purins supplied the final link of a chain of reactions which could thoroughly account for the presence of uric acid in the body. Moreover, the nucleic acids of invertebrates have been found to yield guanin and adenin and certain of the nuclein ferments have been shown to exist in mollusks.[1]

Not only has this nuclein orgin of uric acid found wide acceptance but there has been a tendency to assume that it alone is sufficient to account for all the endogenous uric acid of the organism. That uric acid can be and must be formed from nucleic acid through the agency of the nuclein ferments is scarcely to be doubted but the question still arises, can endogenous uric acid

[1] Mendel and Wells: *Amer. Journ. of Physiology*, xxiii, p. 170.

be produced in any other way? The work of Burian and Schur answers that it can. They show that the relatively large amount of uric acid found in the urine cannot be accounted for by assuming that its sole origin is in the dead leucocytes. Burian later convinced himself, from a study of the output of uric acid from the muscles of man both at rest and under stimulation and from special researches with the surviving muscle of a dog, that the production of uric acid by muscle is a continuous process.[1] The details of Burian's work are not minutely described but he clearly draws the following conclusions:

(1) The perfusion fluid which initially contains no uric acid always takes on this substance when passed through a resting muscle.

(2) When a muscle is stimulated during the perfusion, the purin bases[2] are increased, and in the period of rest just succeeding the working period there is a continual increase of uric acid but not of purin bases.

(3) In the condition of rest the muscle continually gives up uric acid to the blood at the cost of its hypoxanthin. The oxidation of the hypoxanthin to uric acid is brought about by the xanthoõxidase present in muscle: but the ferment must be localized in such a manner that the oxidation involved can only occur just previous to exit from the muscle fiber, for in muscle itself one never finds uric acid but only purin bases. Since the amount of hypoxanthin in muscle, in spite of this giving off of uric acid to the blood, remains on the whole constant, the muscle must continually renew its hypoxanthin.

Burian's work shows that hypoxanthin is formed in muscles principally during stimulation and that the conversion of the hypoxanthin to uric acid goes on continually both while the muscle is at rest and during stimulation. Burian's chief objection to the nuclear origin of muscular uric acid would therefore be a failure of material. Had he chosen to make the examination, he would have found also a failure of ferments. In order

[1] *Zeitschr. f. physiol. Chem.*, xliii, p. 533.

[2] Burian evidently intends the expression "purin bases" to mean hypoxanthin; in fact he occasionally uses the word hypoxanthin in brackets in explanation of "purin bases" and makes no mention of adenin in this connection.

that uric acid may be formed indirectly from nucleic acid through hypoxanthin, the ferment adenase must come into activity: but we shall show that adenase is not present in dog's muscle, therefore the hypoxanthin produced in working muscles cannot be formed from nucleic acid and must originate in some other way.

Schittenhelm[1] is disposed to treat the nuclein metabolism of muscle as perfectly analogous to that of glands and in a measure ignores Burian's excellent results. He worked with ox muscle and found all four nuclein ferments present as in the case with the liver,[2] and in examining human muscle he finds the same four ferments present as is the case with mammalian muscle. It is true that ox muscle contains adenase, but we have not been able to demonstrate the presence of this ferment in the muscle of any other animal species examined (dog, pig, rabbit). Yet the muscle of all of these species constantly contains a relatively large amount of hypoxanthin which in the case of the dog, as certainly shown by Burian and presumably also in the cases of other animal species, is formed as a result of muscle stimulation. This formation of hypoxanthin cannot result from nucleic acid in those animals whose muscles contain no adenase, and it is fair to conclude that even in the case of the ox there is likewise a production of hypoxanthin which is independent of the phagocytes in spite of the fact that this tissue contains the ferments which are capable of forming hypoxanthin from nucleic acid.

For the want of a better term we have chosen to name "preformed hypoxanthin" that hypoxanthin which can be found in a number of tissues and which is not formed from adenin through the disamidizing action of adenase. In the earlier work of this laboratory the tissues examined contained adenase, so that if preformed hypoxanthin were present it was not possible to distinguish this from the much larger amount of hypoxanthin formed from adenin, since the tissues in question were rich in nucleic acid. Again, if a tissue contains xanthoöxidase a small amount

[1] *Zeitschr. f. physiol. Chem.*, xlv, p. 138.

[2] Schittenhelm did not demonstrate the presence of adenase in ox muscle; he proved the presence of guanase and regards guanase and adenase as one and the same ferment. Both ferments are present in ox muscle.

of hypoxanthin would surely be oxidized during self-digestion to xanthin or uric acid and its original presence would not be suspected. In order therefore to be able to prove preformed hypoxanthin a tissue must be examined which contains neither adenase nor xanthoöxidase.[1] The first tissue to be examined which fulfilled those conditions was human spleen.

The presence of adenase is sufficiently shown in a tissue if it be found that adenin disappears when allowed to remain in an aqueous extract of such tissue at the body temperature with the corresponding production of hypoxanthin. The finding of a trace of hypoxanthin after digestion with adenin is of itself no proof of the presence of adenase, for such a conclusion would necessitate the absurd assumption that hypoxanthin can be formed in but this one way. If therefore one should find a trace of hypoxanthin in a digestion experiment after a practically quantitative recovery of the adenin which had been added, he would surely not be justified in assuming the presence in the tissue extract of a "trace" of adenase (for a "trace" of adenase does not decompose a "trace" of adenin if time be allowed) but would more properly suspect that a trace of hypoxanthin may have been present in his specimen of adenin or that some decomposition of adenin had been brought about by the chemical treatment employed in the method of isolation. After considerable trouble we were able to convince ourselves of the truth of the following propositions:

(1) The human spleen contains no adenase.

(2) The specimen of adenin which we employed was free from hypoxanthin.

(3) The method of isolation which we employed exerts no appreciable action on adenin.

(4) A trace of hypoxanthin is always found among the products of the self-digestion of human spleen and by uniting the hypoxanthin fractions of a number of experiments a sufficient amount of material can be obtained for an identification of the base.

[1] At least a tissue so lacking in available oxygen or in which, for other reasons, xanthoöxidase is not capable of effecting the oxidation of hypoxanthin.

After having met a similar experience with other tissues we came to the conviction that all organs contain at least a trace of preformed hypoxanthin. One is reminded of muscle tissue in which hypoxanthin is always found in the absence of its constant and necessary companion in nuclein metabolism: a tissue moreover which is peculiarly poor in nucleic acid. We were easily able to convince ourselves that the voluntary muscles of the pig, dog and rabbit contain no adenase but that in these tissues is always to be found a relatively large amount of hypoxanthin. This observation offers a more or less probable explanation of the trace of hypoxanthin found in various organs. All organs are supplied with involuntary muscle, and Saiki has shown that while guanin and adenin can be found in traces due evidently to the decomposition of a small amount of nucleic acid, yet, as is the case with striated muscle, the conspicuous base of non-striated muscle is hypoxanthin.[1] It therefore seems true that the most important purin base which contributes to the endogenous uric acid is muscular preformed hypoxanthin and that this preformed hypoxanthin is not directly connected with the nuclein metabolism, since it may occur in the absence of adenase, an essential factor in the passage from nucleic acid to hypoxanthin.

EXPERIMENTAL PART.

The muscles of the pig and ox which were used in the following experiments were obtained from the slaughter house, those of the rabbit from the physiological laboratory, the animals having received anæsthetics. This however, as long experience has taught us, can make no difference in the exhibition of the nuclein ferments of other tissues. But the dog's muscle was dissected from living animals under deep anæsthesia, ground immediately and made into a paste with twice its weight of water. After almost continual agitation for an hour with the use of chloroform as a preservative, the solution was strained off and the residue was extracted in the same way with two parts of water. The two aqueous extracts were well mixed, a portion removed for a check test and the remainder used in all experiments described. The experiments thus having been made with the same speci-

[1] This *Journal*, iv, p. 483.

men of muscles, extracts are strictly comparable with one another. The adenin which was added was a carefully prepared specimen of adenin sulphate which had been found completely precipitable by picric acid, giving a filtrate which formed no precipitate with silver nitrate and ammonia, and was therefore free from hypoxanthin. Before addition to the muscle extract the adenin sulphate was weighed out into a glass dish, suspended in hot water and brought into solution with the smallest possible amount of caustic soda. In the experiments where only adenin or hypoxanthin came under consideration the following very convenient departure from the usual method of isolation was employed. The products of digestion were made very faintly acid with acetic acid, and the proteids coagulated by heat and filtered off. The pale-yellow filtrate was allowed to cool and treated with basic lead acetate in successive portions until a small portion of the fluid was found to give a decided precipitate with sulphuretted hydrogen. The lead precipitate was then filtered off and the excess of lead removed with sulphuretted hydrogen. After evaporation to half its volume on the water bath the fluid was examined for hypoxanthin and adenin in the usual way. The introduction of basic lead acetate as a reagent makes a considerable difference. A fluid is finally obtained free from phosphates and as colorless as water, while there is not the slightest danger of losing either of the bases in question through the use of the reagent.

1. Ox Muscle.

Extract of 523 grams of muscle, 1 : 4; without digestion:
Hypoxanthin nitrate obtained ..... 0.366 gram
Hypoxanthin nitrate for one gram of muscle..... 0.00070 "

Extract of 531 grams of muscle 1 : 4; without digestion:
Hypoxanthin nitrate obtained..... 0.345 gram
Hypoxanthin nitrate for one gram of muscle..... 0.00065 "

Extract of 415 grams of muscle, 1 : 4; digestion 6 days:
Hypoxanthin nitrate obtained..... 0.297 gram
Hypoxanthin nitrate for one gram of muscle..... 0.00071 "

Extract of 504 grams of muscle, 1 : 4; digestion 6 days:
Hypoxanthin nitrate obtained..... 0.340 gram
Hypoxanthin nitrate for one gram of muscle..... 0.00067 "

The hypoxanthin of ox-muscle extract does not increase in autodigestion.

Extract of 174 grams of muscle, 1 : 4:
Adenin sulphate added 0.300 gram; digestion 4 days.
Hypoxanthin nitrate found.....0.420 gram
Hypoxanthin nitrate from the muscle.....0.117 "
Hypoxanthin nitrate from added adenin....0.303 " or 95 per cent

Extract of 255 grams of muscle, 1 : 4:
Adenin sulphate added 1.000 gram; digestion 4 days.
Hypoxanthin nitrate obtained.....1.178 grams
Hypoxanthin nitrate from muscle.....0.179 gram
Hypoxanthin nitrate from adenin.....0.999 " or 94 per cent

Ox muscle contains adenase.

Extract of 255 grams of muscle, 1 : 4:
Guanin sulphate added.....1.000 gram; digestion 4 days
Xanthin nitrate obtained.....0.800 " or 80 per cent
No guanin found.

Ox muscle contains guanase.

2. Dog's Muscle.

Extract of 600 grams of muscle, 1 : 4; digestion 3 days
Hypoxanthin nitrate found.....0.385 gram
Hypoxanthin nitrate for one gram of muscle.0.00064 "

Extract of 80 grams of muscle, 1 : 4:
Adenin sulphate added.....0.350 gram; digestion 6 days
Adenin purate obtained.....0.602 " or 96 per cent

Dog's muscle extract contains hypoxanthin but cannot change adenin into hypoxanthin.

Extract of 250 grams of muscle, 1 : 4:
Adenin sulphate added, 0.600 gram. After digestion for 2 days half of the material removed and the remainder allowed to digest for 25 days.
(a) Digestion 2 days:
Hypoxanthin nitrate.....0.083 gram
Adenin picrate.....0.50 " or 96 per cent
(b) Digestion 25 days:
Hypoxanthin nitrate.....0.072 gram
Adenin picrate.....0.555 " or 1 00 per cent

The hypoxanthin is no greater and the adenin no less, after a digestion of 25 days than after a digestion of 2 days.

3. Rabbit's Muscle.

Extract of 85 grams of muscle, 1 : 4:
Adenin sulphate added.....0.350 gram; digestion 6 days
Hypoxanthin nitrate found.....0.065 "
Adenin picrate.....0.540 " or 86 per cent

Rabbit's muscle extract contains hypoxanthin but cannot convert adenin into hypoxanthin.

4. Pig's Muscle.

Extract of 85 grams of muscle, 1 : 4:
Adenin sulphate added.....0.350 gram; digestion 6 days
Hypoxanthin nitrate found.....0.600 "
Adenin picrate.....0.601 " or 97 per cent.

Thus the muscles of the pig, dog and rabbit have no power of converting adenin into hypoxanthin yet contain hypoxanthin. So far as concerns nuclein metabolism, the ox muscle resembles a gland; it possesses the ferments capable of decomposing the purin of nucleic acid, but the material for these transformations is not present. On the other hand ox muscle contains hypoxanthin, and all things considered we are justified in concluding that this is preformed hypoxanthin. The adenase and guanase of ox muscle must therefore for the present find a place in the large and increasing group of useless functions and may with some justification be looked upon as rudiments. In any event muscular tissue is not the seat of nuclein metabolism.

THE INTRACELLULAR ENZYMES OF LOWER FUNGI, ESPECIALLY THOSE OF PENICILLIUM CAMEMBERTI.

By ARTHUR WAYLAND DOX.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

(Received for publication, July 20, 1909.)

The renewed interest which has been manifested in recent years in the study of enzymes has made timely a reinvestigation of the intracellular enzymes of certain groups of lower plants which play important rôles in various biochemical processes. It is not desired to review in this place the extensive literature on the enzymes of molds which is scattered in various chemical, biological, botanical and mycological journals. It is proposed to publish in a bulletin of the Bureau of Animal Industry of the U. S. Department of Agriculture, and also in the biennial report of the Storrs Agricultural Experiment Station for 1909,a detailed résumé of the earlier researches in this domain, in connection with a more elaborate report of the studies to which reference is made in the present summary.

In the studies on the ripening of soft cheeses which have engaged the writer's[1] attention for several years, it has become evident that a detailed analysis of the enzymatic reactions induced by the molds which are the primary factors in the manufacture of these products is of considerable importance. The data at present on record are conflicting and inadequate to explain many of the characteristic phenomena of cheese "ripening." Attention has therefore been directed to the equipment of the essential molds in enzymes. Since it is now well recognized that many of these organisms do not secrete their enzymes readily into the surrounding medium, special consideration has been devoted to the so-called endo- or intracellular enzymes.

At the outset it may be noted that the enzymatic characteristics of these lower organisms are modified to no inconsiderable

[1] Dox: Proteolytic Changes in the Ripening of Camembert Cheese. Bulletin 109, B. A. I., U. S. Dept. of Agriculture.

extent by the character of the culture medium in which they develop. This is particularly pronounced in the case of those activities associated with carbohydrates. The changes in content of enzyme are, however, essentially quantitative in character. There is no evidence that enzymes not normally formed by the organism in demonstrable quantities can be developed by special methods of nutrition. The influence of a particular substrate appears therefore to be in the direction of stimulating the production of an enzyme which is normally found under all conditions—not to develop an entirely new enzyme.

A part of the confusion at present existing regarding the enzymes of molds is unquestionably due to the confusion respecting the nature of the organisms employed. The writer has therefore placed emphasis on the desirability of working with well-identified fungi grown under known conditions. For special reasons, *Penicillium camemberti* has been selected for the preliminary studies. Its characteristics are well known, and its economic importance specially commended the use of this mold.

Preparation of the Enzyme. The following methods of growing the fungus and preparing the enzyme-yielding products have proved to be particularly effective. A modification of the medium used by Czapek[1] was found to yield vigorous cultures of the organism. As used in these experiments its composition is as follows:

Water	1000.0	cc.
Magnesium sulphate (cryst)	0.5	gram
Potassium acid phosphate	1.0	"
Potassium chloride	0.5	"
Ferrous sulphate	0.01	"
Sodium nitrate	2.0	grams
Cane-sugar	30.0	"

The medium is placed in a number of large Erlenmeyer flasks to the depth of about 3 cm. The mouth of each flask is closed with a cotton plug and the flasks sterilized in an autoclave. Inoculations are made from a pure culture by means of a sterile platinum wire. The cultures are allowed to develop in diffuse daylight at laboratory temperature until about the tenth day,

[1] Hofmeister's *Beiträge*, i, p. 542.

when the bluish-green color of the spores begins to appear. The mold is then removed and treated according to Albert and Buchner's[1] method for "Acetondauerhefe." As adapted to molds the method is as follows: The whole mycelium is immersed for a few moments in running water, then squeezed to remove as much as possible of the water, and run through a hashing machine. The wet mass thus obtained is immersed for ten minutes in a large volume of acetone, with constant stirring, then it is filtered off with suction. It is now immersed again for two minutes in a fresh quantity of acetone and filtered as before. The third time it is immersed in ether for three minutes, sucked as dry as possible, and spread out in a thin layer for several hours until the odor of ether is no longer perceptible. A coarse dry powder results, which is ground still finer by a suitable grinding machine.

This powder contains, besides the enzymes, all the constituents of the cells that are not soluble in acetone and ether. When kept out of the light and in well-stoppered bottles, the enzymes in this form seem to retain their activity indefinitely. A specimen more than a year old is apparently as active as it was when freshly prepared. For enzyme experiments the powder may be used in suspension or in the form of a filtered aqueous extract.

Proteases. Despite the conflicting statements in the literature regarding the effect of mold enzymes upon simple proteins such as fibrin and ovalbumin, it is currently believed that the proteolytic agent corresponds with ordinary trypsin. In the present study we have been unable to demonstrate any proteolytic activity on the part of the intracellular enzymes of *P. camemberti* toward fibrin, ovalbumin, or other native proteins. On the other hand, casein, gelatin and proteoses (in the form of Witte peptone) are readily digested. In this respect the enzyme is closely related to the erepsin discovered by Cohnheim in the intestine of mammals. Its activity is greatest in media neutral or faintly acid with phosphates. The exception which casein affords in its susceptibility to digestion by the protease of *P. camemberti* has been verified in the case of *P. chrysogenum* also, and corresponds with the well-known characteristic of animal erepsin. It is obviously of peculiar interest in connection with cheese ripen-

[1] *Ber. d. deutsch. chem. Ges.*, xxxv, p. 2376.

ing, since casein is the essential proteolytic substrate on which the organism reacts. A typical illustration of this peculiar relation is shown in the following protocols, where the enzyme was allowed to act under comparable conditions upon 0.1 gram portions of various proteins. The unique position of casein is at once apparent.

PROTEIN ADDED	UNCHANGED PROTEIN RECOVERED, GRAMS.		
	$\frac{N}{20}$ HCl	$\frac{N}{20}$ Na_2CO_3	Neutral
Zein	0.0996	0.0987	0.0998
Cryst. edestin	0.0993	0.0987	0.0989
Excelsin	0.0991	0.0970	0.0990
Elastin	0.1002	0.0980	0.0997
Collagen	0.0996	0.1005	0.0991
Ovovitellin	0.0982	0.0974	0.0978
Cocoanut protein	0.0982	0.0990	0.0991
Casein	0.0876	0.0323	0.0006
Casein (control)	0.0980	0.1003	0.0994

When proteose solutions are treated with the enzyme, the compounds yielding a biuret reaction are rapidly changed and the tryptophan reaction with bromine water simultaneously develops. The gelatin digesting power is likewise characteristic for animal erepsin.

Despite wide variations in the character of the synthetic media upon which the enzyme-producing molds were grown, erepsin was uniformly found present in every product investigated.

Nuclease. The preparations contain a nuclease capable of liberating purines and phosphoric acid from the nucleic acid of yeast. The enzyme is developed independently of the presence of nucleic acid or organic nitrogen compounds in the culture medium. In illustration of this reaction the following protocol is offered. The digestion was carried on for forty-five days in the presence of toluene, and the figures represent grams per 100 cc.

	gram
Total nitrogen	0.1788
Purine nitrogen	0.0663
Total phosphorus	0.0859
Phosphorus liberated	0.0512

Control, boiled enzyme.

Total nitrogen	0.1788
Purine nitrogen	0.0036
Total phosphorus	0.0859
Phosphorus liberated	0.0041

Amidases. The following illustrative protocol will suffice to indicate the power of the enzyme to liberate ammonia from appropriate compounds.

SUBSTRATE (0.2 gm)	TIME	N LIBERATED AS NH_3
	days	*grams*
Urea	4	0.0174
Asparagine	4	0.0144
Biuret	5	0.0006
Acetanilide	5	0.0000
Alanine	5	0.0015
Benzamide	5	0.0021

Enzyme acting on hippuric acid. Enzyme preparations from several molds (*P. camemberti, P. chrysogenum, P. brevicaule* and *Aspergillus niger*) were found to contain an enzyme which acts vigorously on hippuric acid, hydrolyzing it into benzoic acid and glycocoll. This hydrolytic reaction is apparently specific for these enzyme preparations, inasmuch as Cohnheim[1] failed to obtain a comparable reaction with very active erepsin preparations of animal origin.

Emulsin. The three typical glucosides, amygdalin, salicin and arbutin, are readily decomposed by the intracellular enzymes present in molds cultivated on a medium containing a carbohydrate only as the source of carbon. Phlorhizin is somewhat less readily acted upon.

Lipase. The feeble reactions obtained when the enzyme powder was tested with various esters, such as triacetin, monobutyrin, amyl acetate, ethyl acetate and ethyl butyrate, may be attributable to the absence of lipoid material in the culture media employed, or to the technique of isolation applied. It is known that lipases are soluble in ether containing lipoid material,[2] and

[1] Cohnheim: *Zeitschr. f. physiol. Chem.*, lii, p. 526.

[2] Taylor: This *Journal*, ii, p. 87.

possibly lipase has been removed or destroyed in the process of isolation employed by us. Preliminary experience regarding the growth of mold itself in the presence of butter fat and the obvious hydrolysis of the latter lead the writer to withhold a final conclusion as to the presence of lipase among the endoenzymes of the mold until more convincing experiments have been concluded.

Enzymes acting on carbohydrates. The study of the enzymes acting on carbohydrates has been somewhat complicated by the presence of carbohydrate in the mold itself. Aside from the hemicelluloses, polysaccharides closely related to glycogen may be detected. Mannite has also been isolated. It soon became apparent that under conditions in which autolysis of the mold could proceed reducing sugars were liberated which interfered with the interpretation of the results of our experimental trials. The errors from this source were reduced to a minimum by using very small quantities of the enzyme preparation for the digestion trials and by making control autodigestion experiments. It is also possible to eliminate this source of error by subjecting the autodigested enzyme solutions to dialysis to remove the sugars; although the enzyme itself is somewhat diminished in activity by this procedure, most likely owing to a destructive action of the protease present or possibly to slight diffusion of the enzyme itself.

The carbohydrate digesting enzymes thus far specifically identified in *P. camemberti* are amylase, inulase, raffinase, sucrase maltase and lactase. As already stated, the influence of the composition of the medium in respect to the carbohydrates present on the resulting content of these enzymes in the mold is of striking interest. The numerous details in evidence of this will be presented in the subsequent report. Inulase affords the most conspicuous example, and the following protocol will be taken for illustration.

Enzyme preparations from different culture media as indicated were allowed to act on a 2 per cent solution of inulin for forty-two hours. The solution was then clarified and the rotation determined.

CULTURE MEDIUM	ROTATION ° VENTZKE	HYDROLYSIS PER CENT
Sucrose	−2.6	3
Maltose	−2.6	3
Lactose	−2.6	3
Starch	−2.6	3
Glucose	−2.6	3
Inulin	−5.9	92
Control	−2.5	0

The amylase reactions were studied with starch paste, soluble starch (amidulin) and dextrins. Glycogen appears to be attacked with difficulty if at all. In correspondence with this it is interesting to note that *P. camemberti* fails to grow upon a synthetic medium containing glycogen as its source of carbon, although the starch derivatives mentioned above are very effective as culture media. This observation has a bearing upon the existence of a specific glycogenase maintained by some writers.

The study of the chemical compounds of cheeses in the preparation of which molds of the type of *P. camemberti* are employed has shown the absence of compounds such as indol, mercaptan, hydrogen sulphide and phenol, which are commonly associated with so-called "putrefactive" changes. All of the compounds thus far identified by the writer in these products can be ascribed to digestive changes which the enzymes discussed in the preceding pages are themselves able to effect upon the milk component originally entering into the product. The bearing of these facts upon the theories of cheese ripening is obvious.

The writer wishes to acknowledge his indebtedness to Prof. Lafayette B. Mendel for valuable advice and assistance throughout this investigation, and to Dr. Charles Thom, of the Bureau of Animal Industry, for the mold cultures.

THE PURINES AND PURINE METABOLISM OF THE HUMAN FETUS AND PLACENTA.[1]

By H. GIDEON WELLS and HARRY J. CORPER.

(*From the Pathological Laboratory of the University of Chicago.*)

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In the study of the development of the several enzymes involved in purine metabolism in the embryo pig, Mendel and Mitchell[2] found that these several enzymes do not appear together, but one at a time, and some not until late in antenatal or early in postnatal life. Even at the earliest stages studied, 50 mm. embryos, the enzymes which liberate the purines from the nucleoproteins are present, the *nuclease* of Ivanoff. The deamidizing enzymes are also present at a correspondingly early stage, *adenase* being demonstrated by the reduction of the quantity of adenine and increase in hypoxanthine in autolyzing livers from pigs of 50 mm., 75 mm., and 100 mm. length.[3] *Guanase* is absent from the liver of the pig at all times, in the adult as well as in the fetal animal, but it could be demonstrated in the other viscera of embryo pigs of 120 mm. length, the smallest examined for this enzyme, by the conversion of guanine into xanthine by extracts of the combined organs. The next step in purine metabolism is the conversion of hypoxanthine and xanthine into uric acid by a process of oxidation, which is accomplished by an oxidizing enzyme or enzymes, the *xantho-oxidase*

[1] This work has been aided by a grant from the Rockefeller Institute for Medical Research.

For materials we are indebted to several Chicago physicians, especially to Dr. J. W. Jobling of the Michael Reese Hospital. Much assistance in the analytical work has been given by Mr. J. H. Mitchell.

[2] *Amer. Journ. of Physiol.*, xx, p. 97, 1907.

[3] Jones and Austrian (this *Journal*, iii, p. 227, 1909) could find no evidence of adenase in the liver until pig embryos have reached a length of 150 to 170 mm.

of Burian. This enzyme could not be detected in the livers or other viscera of embryo pigs up to 230 mm., the largest studied, but was present in the liver of a sucking pig about seven weeks old; intermediate stages were not examined, so the exact time of appearance of this enzyme cannot be stated. As the last step in purine metabolism in the pig, the uric acid formed by the other enzymes is destroyed by yet another oxidizing enzyme, the *uricase* of Battelli and Stern.[1] This enzyme is also missing in the liver or other tissues of fetal pigs of 200 mm. or less, and seems to be but feebly developed in the liver of sucking pigs two months old, although very active in the liver of adult pigs. Mendel and Mitchell remark "that the tardy appearance of the oxidative and katabolic enzymes concerned in the transformation of the purines is suggestive as a characteristic of growing, synthetic organs."

This development of the enzymes of purine metabolism, step by step, furnishes an interesting demonstration that the biological law, that the individual in its development reflects the entire developmental history of the species to which it belongs, may hold just as true in biochemistry as in morphology. The purine enzymes of the pig at different stages of development reflect the conditions in different forms of life, for the simplest forms would seem, as far as the investigations yet made show us, to have a correspondingly simple outfit of purine enzymes. As the scale of complexity increases, the number of these enzymes increases until in the adult mammal we have the maximum.[2] Thus in the yeast there is nuclease, as shown by the appearance of free purines during autolysis of yeast, and also a single deamidizing enzyme, guanase, but no adenase or xantho-oxidase.[3] An invertebrate organism, the mollusk *Sycotypus canaliculatus*, has been shown to possess nuclease, guanase, and adenase, but no xantho-oxidase or uricase.[4] The embyro pig at 200 mm. or less is, therefore, equipped with a scanty number of purine enzymes, similar to the yeast or the mollusk. When later it

[1] *Compt. rend. soc. biol.*, Paris, lxvi, p. 612, 1909.

[2] Compare Herlitzka: *Arch. ital. de biol.*, xlviii, p. 119, 1907, "Sur l'ontogénèse des ferments."

[3] Straughn and Jones: This *Journal*, vi, p. 245, 1909.

[4] Mendel and Wells: *Amer. Journ. of Physiol.*, xxiv, p. 170, 1909.

acquires the xantho-oxidase, but is still lacking the uricase, it would seem to be in the same condition as the birds and reptiles, which form uric acid but seem to be entirely unable to destroy it. The uricolytic property is a later development, and in man this would seem never to be acquired, or at least not actively,[1] in which respect man is behind the cow, dog, rabbit and guinea-pig, all of which animals can destroy uric acid readily.[2]

In the following article it will be shown that the sequence of enzyme formation in the human fetus is quite similar to that observed by Mendel and Mitchell and by Jones and Austrian in the fetal pig.

ADENASE AND GUANASE.

The mature human fetus possesses both adenase and guanase, as shown by the following experiments:

Experiment I.—A full-term female fetus, weight 3400 grams, which died at birth because of lack of attendance. After removing the viscera for use in uricolysis experiments, the rest of the fetus, weighing 1835 grams, was ground up and allowed to autolyze in 5500 cc. water, after adding the solid residues of the viscera left after extracting them over night in water, and straining through muslin. (The extrats were used in uricolysis experiments.) Autolysis was continued for 38 days at 36–38° C., without access of air to the autolyzing mixture which was covered by a thick layer of toluol. The mixture was found to be distinctly acid to litmus in spite of the presence of the bone salts. The coagulable proteins were removed by coagulation and filtration, and the purines were precipitated by the copper sulphate and sodium bisulphite method of Krüger and Solomon. The purines were freed with hydrogen sulphide and fractionated in the usual way. There was no purine present that was not readily soluble in 1 per cent ammonia solution, showing the absence of *guanine*, but there was a large amount of purine coming out in crusts and scales which was insoluble in a considerable volume of water at 35° C. After being purified this material gave the characteristic reaction of *xanthine*, and was entirely free from uric acid. The amount of xanthine recovered was 0.7 gram.

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[1] See Wells and Corper: This *Journal*, vi, p. 321, 1909.

[2] According to Jones and de Angulo (*Proc. Amer. Soc. Biol. Chemists, i*, p. 193, 1909) the entire equipment of purine enzymes in the dog's liver is acquired only after birth, not even guanase or adenase being present in newly born dog's liver.

The filtrate from the xanthine did not give any precipitate with picric acid, showing the absence of adenine. It gave, however, an abundant precipitate with ammoniacal silver chloride, which on being redissolved in hot dilute nitric acid gave a large crop of typical crystals of hypoxanthine silver nitrate, weighing 0.712 gram after drying at 120° C. This corresponds to 0.266 gram of *hypoxanthine*.

The presence of such an abundance of free xanthine and hypoxanthine in the autolyzing mixture and the absence of guanine and adenine indicates the presence of both *guanase* and *adenase*. It would also seem that guanine is more abundant than adenine in the fetal tissues, in view of the relative excess of xanthine over hypoxanthine.

Studies of earlier stages show an interesting condition, namely, that the adenase and guanase do not appear at the same time, the guanase being found earlier than the adenase. This is shown by the two following experiments with fetuses of almost identical age:

Experiment II.—A fetus of about the third month, 8 cm. long, weight 9 grams, was ground up, and to it was added in solution 0.039 gram adenine hydrochloride (0.029 gram adenine) and 0.045 gram guanine hydrochloride (0.034 gram guanine). Autolysis was continued 37 days at 37° C., without air. At the end of that time, when analyzed, the guanine had entirely disappeared, being replaced by *xanthine* of which 0.014 gram was recovered. On adding picric acid to the filtrate from the guanine a voluminous precipitate of typical adenine picrate was obtained, which had a melting point of 273° (uncorr.), and weighed 0.057 gram, corresponding to 0.021 gram *adenine*. After removal of the adenine picrate the filtrate was found to still give a small precipitate with copper sulphate and sodium bisulphite. On decomposing with hydrogen sulphide and reprecipitating with ammoniacal silver chloride a slight flocculent precipitate was obtained indicating the presence of a trace of some purine, but the amount was too small to identify; presumably it was either a trace of hypoxanthine derived from the fetal tissues or traces of one of the other purines which had failed to be removed in the earlier steps of the analysis.

This experiment indicates that in the fetus at three months guanase is present, but adenase is still absent. It is well corroborated by a duplicate experiment with a fetus of almost exactly the same size.

Experiment III.—A three-months fetus, obtained by operation from a freshly ruptured tubal pregnancy. Length 6.5 cm., weight 8 grams. Ground up and placed in a solution containing 0.0962 gram guanine hydrochloride (0.074 gram guanine) and 0.1041 gram adenine hydrochloride

(0.078 gram adenine). Let autolyze without air for 13 days at 37°. Recovered 0.046 gram *xanthine*, no guanine, and 0.1501 gram adenine picrate (corresponding to 0.056 gram *adenine*) with a melting point of 270°(uncorr.). After removal of the adenine picrate there was still a minute amount of purine precipitable by copper sulphate, which was freed from copper with hydrogen sulphide, reprecipitated with ammoniacal silver chloride, and recrystallized after solution in boiling, dilute nitric acid. A few milligrams (0.029 gram) of hypoxanthine silver nitrate crystals were obtained, corresponding to about 10 mg. of *hypoxanthine*.

These two experiments furnished convincing evidence of the independence of the two deamidizing enzymes, adenase and guanase, which was disputed by Schittenhelm but may be now considered to be conclusively settled by the results obtained by Jones and Mendel and their co-workers.[1]

Between the third and fifth months the adenase makes its appearance, as shown by the next experiments.

Experiment IV.—Fetus about fifth month, 26 cm. long, weight 450 grams, weight of liver 27 grams. Made separate emulsions of the ground-up liver and the remainder of the fetus, and to each added guanine hydrochloride and adenine hydrochloride. Autolysis was continued at 37°, without air, for 50 days, and then the free purines were isolated. It was found that the adenine and the guanine had both entirely disappeared, their place being taken by xanthine and hypoxanthine, the results of quantitative determinations being as follows:

(A) Liver, weight 27 grams. Added 0.1080 gram adenine hydrochloride (0.080 gram adenine) and 0.080 gram guanine hydrochloride (0.061 gram guanine). Recovered 0.0596 grams *xanthine* and 0.0688 gram *hypoxanthine* (0.1841 gram hypoxanthine silver nitrate).

(B) Fetus, weight 420 grams. Added 0.1105 gram adenine hydrochloride (0.0825 gram adenine) and 0.1230 gram guanine hydrochloride (0.0947 gram guanine). Recovered 0.1524 gram *xanthine* and 0.118 gram *hypoxanthine* (0.3165 gram hypoxanthine silver nitrate).

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[1] It may be mentioned that Schittenhelm himself has obtained excellent evidence of the existence of two separate deamidizing enzymes acting respectively on adenine and guanine, for he reports (Schittenhelm and Schmidt, *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 428, 1907) that the lung of the human fetus has the power of converting guanine into xanthine, although it does not convert adenine into hypoxanthine (Expts. 15 and 16). Apparently he failed to appreciate the significance of this result, since a few pages later (pp. 432–437) he tries to show that Jones is in error in maintaining the existence of two separate enzymes.

These results indicate a conversion of the adenine and guanine added to the liver to hypoxanthine and xanthine which is practically quantitative; in the case of the entire fetus there has been some gain in the xanthine and hypoxanthine, presumably from autolysis of the 420 grams of tissue that were present in the mixture.

Experiment V.—Male fetus, about the fifth month, length 25 cm., weight 235 grams. This was ground up, put in one liter of water and toluol to which had been added 0.0986 gram adenine hydrochloride (equivalent to 0.072 gram adenine) and autolysis continued without air for 12 days at 37°. The free purines were precipitated and isolated by the usual method. No guanine was found, but a trace of what seemed to be xanthine. Little or no adenine remained, since but a faint turbidity was produced with picric acid, and that only after standing for some time. The chief purine obtained was *hypoxanthine*, which, isolated as the hypoxanthine siver nitrate, weighed 0.1752 gram, corresponding to 0.0654 gram hypoxanthine. Evidently, therefore, the adenine has been converted into hypoxanthine.

Experiment VI.—A female fetus, weight 605 grams, length 32 cm., probably about the sixth month. The entire tissue was ground up and permitted to autolyze for 43 days under toluol, without air, in 2500 cc. of water, about half the time at 37° and the rest of the time at room temperature. After removal of the insoluble and coagulable materials the purines were precipitated and isolated in the usual way. No guanine, uric acid or adenine was recovered, but there was obtained 0.283 gram *xanthine* and 0.412 gram hypoxanthine silver nitrate, corresponding to 0.154 gram *hypoxanthine*.

These last three experiments establish the presence of both *adenase* and *guanase* in the liver and other tissues of the human fetus at the fifth and sixth months. Experiment IV also proves that the human liver possesses both these enzymes, in contrast to the liver of the pig which has only adenase, and the liver of the dog which has only guanase.[1]

XANTHO-OXIDASE.

The following experiments show that, as in the pig, the oxidizing enzyme appears later than the deamidizing enzymes. but yet some time before birth of the human fetus.

[1] Jones and de Angulo: *loc. cit.*

Experiment VII.—Fetus, said to be 8½ months, but the centers of ossification at the end of the femur were about 4 mm. in diameter. Length 48 cm., weight 2745 grams. Died at birth from intra-abdominal hemorrhage. The various viscera were ground up separately, extracted over night at room temperature, strained, and after addition of 0.1 gram xanthine to each, permitted to autolyze for 48 hours at 37° with a current of air bubbling slowly through all the time. Analysis of the mixture after this time gave the following results:

	Tissue.	Material added.	Material recovered.
1.	Liver (168 gm.)	0.1 gm. xanthine	0.1565 gm. uric acid.
2.	Muscle (109 gm.)	0.1 " "	0.0952 " xanthine.
3.	Intestine (41 gm.)	0.1 " "	0.0963 " "
4.	Kidneys (20 gm.)	0.1 " "	0.1013 " "
5.	Spleen and Thymus (13 gm.)	0.1 " "	0.0914 " "

From this experiment it would seem that in a fetus just before birth there is present in the liver an oxidizing enzyme which produces uric acid not only from the xanthine added to it, but also apparently from the purines of the liver itself. The other viscera and the muscle seem to be devoid of xantho-oxidase.

Experiment VIII.—A full-term infant, which lived but three hours on account of a congenital heart defect. The liver (weight 200 grams), the kidneys (weight 22 grams), and the remaining viscera (total weight 190 grams), were ground, extracted over night, and strained. The liver and viscera extracts were divided into equal parts, and after adding uric acid or xanthine as indicated below, the five extracts were allowed to autolyze at 37° for 48 hours with a constant current of air bubbling through. The results of analysis of the resulting mixtures were as follows:

	Tissue	Substance added	Recovered	Per cent recovered
1.	Liver (100 gm.)	0.1514 gm. uric acid	0.2002 gm. uric acid	132
2.	Liver (100 gm.)	0.0958 " xanthine	0.0856 " " "	
3.	Viscera (95 gm.)	0.1514 " uric acid	0.1706 " " "	112
4.	Viscera (95 gm.)	0.0958 " xanthine	0.0463 " " " 0.0687 " xanthine	
5.	Kidney (22) gm.)	0.1514 " uric acid	0.1488 " uric acid	98

This experiment demonstrates that the liver and at least part of the viscera of the full-term fetus contain xantho-oxidase. The reason for the incompleteness of the conversion of the xanthine into uric acid by the mixed viscera may be the power of one

tissue to destroy the oxidizing enzymes of another, as observed by Künzel and Schittenhelm.[1] The absence of uricolytic enzymes is also shown by this experiment.

Experiment IX.—A well-developed, full-term normal male fetus, weight 3000 grams, which had been kept in an ice chest 36 hours, was ground up and let autolyze at 36° with air current, in 7000 cc. water and toluol. Autolysis was continued 23 days, the air current being run through for a day at a time at intervals. The free purines were separated as usual. No guanine or uric acid or adenine found, but 1.05 gram was of *xanthine* and 0.91 gram hypoxanthine silver nitrate, or 0.34 gram *hypoxanthine*.

Presumably the failure to demonstrate xantho-oxidase in this experiment depends upon the fact that this enzyme is very labile, and by the time the purines had been split out of the tissues, already dead 36 hours before autolysis began, the xantho-oxidase had been rendered inert. Also, as mentioned previously, the xantho-oxidase of one organ may be destroyed by the enzymes of another organ; hence experiments in which all the tissues are combined, as in this and the two following experiments, are of doubtful value.

Experiment X.—A fetus of about the third or fourth month, measuring 11 cm. and weighing 32 grams, was ground up, extracted with toluol-water over night at room temperature, and the extract strained through muslin. To it was added 0.0765 gram xanthine in solution, and it was permitted to autolyze under toluol with a constant stream of air bubbling slowly through for 48 hours. The free purines were then isolated in the usual manner, and found to consist chiefly of *xanthine*, of which 0.046 gram was recovered. No uric acid could be recovered, or even detected by the murexide test in the combined purines.

Experiment XI.—A fetus apparently between the fourth and fifth month, measuring 23 cm. long, and weighing 306 grams, was used for testing the presence of uricolytic enzymes and xantho-oxidase. It was ground up, autolyzed over night at room temperature, strained, and the extract divided into equal parts. Added 0.1445 gram uric acid to one (A) and to the other (B) 0.1405 gram xanthine. Let autolyze with air current for 48 hours, and recovered from A 0.1443 gram uric acid and from B 0.1407 gram xanthine. Therefore at this stage of development there seems to be neither xantho-oxidase nor uricolytic enzymes.

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[1] *Zeitschr. f. exp. Pathol.*, p. 393, 1908.

In the next experiment the difficulty caused by the presence of several viscera in one mixture was avoided, at least as far as the liver is concerned, but the results were still negative.

Experiment XII.—Two fetuses, each about the sixth or seventh month, were obtained in a frozen condition, having been dead and frozen about 48 hours.

(A) weighed 1170 grams, length 37 cm., liver weighed 50 grams.

(B) weighed 755 grams, length 33 cm., liver weighed 40 grams.

The two livers were ground, united, extracted over night with toluol-water, strained through muslin, and to the extract 0.1012 gram xanthine was added. This mixture was autolyzed at 37° with a current of air for three days, at the end of which time 0.1077 gram xanthine was recovered, indicating the absence of active xantho-oxidase, and apparently a slight formation of xanthine from the purines of the liver extract.

United the remaining tissues of the two fetuses, weighing together 1835 grams, and let autolyze 26 days, with an air current passed through the mixture at intervals. Upon analysis of the free purines present at the end of autolysis, guanine, uric acid and adenine could not be demonstrated, but 0.52 gram xanthine and 0.65 gram of hypoxanthine silver nitrate (equal to 0.243 gram hypoxanthine) were recovered.

From these experiments it would seem that xantho-oxidase is present in the liver, possibly in other viscera, but not in the muscle, intestines, kidneys, spleen and thymus, at or shortly before birth. This enzyme seems to make its appearance after the sixth month of intra-uterine life, and before full term. Evidence is also obtained of the lability of the xantho-oxidase, as already demonstrated by Künzel and Schittenhelm, whereby it is readily destroyed during autolysis, and also by the presence of more than one organ in the extracts used for experiments.

PURINES AND PURINE METABOLISM OF THE PLACENTA.

As the fetus is so imperfectly equipped with purine enzymes in the earlier stages of its development, and has at no time the power of destroying the uric acid that its xantho-oxidase may form before birth,[1] it is of interest to consider what supplementary activities the placenta may have to help out the metabolism

[1] See the previous paper by Wells and Corper, this *Journal*, vi, p. 321, 1909.

of the fetus. The old custom of looking upon the placenta as merely a membrane through which substances were passively diffused, has been superseded by a recognition of the important metabolic activity of the placenta, which properly takes rank as an important, actively functionating organ. This view is emphasized in a recent article by Bergell and Falk,[1] in which it is shown that the placenta contains an intracellular enzyme capable of splitting tyrosine out of protein. Other investigations have shown that the placenta also possesses an enzyme or enzymes capable of synthesizing and hydrolyzing glycogen (Hoffbauer), and enzymes which split glucosides and esters (Higuchi).[2]

As a first step an analysis of fresh placenta was made, with particular reference to the quantity and nature of the purines present in this tissue.

ANALYSIS OF PLACENTA.

Three fresh human placentas, weighing 1530 grams together, were ground fine and placed in 7.5 liters of 6 per cent sulphuric acid for two days, until analysis could be done, the material being well mixed with acid to prevent autolysis. The water and total nitrogen were determined in two samples, with the following results:

NO.	FRESH WEIGHT	DRY WEIGHT		NITROGEN	
	gm.	*gm.*	*per cent*	*gm.*	*per cent of fresh weight*
a.....	0.6607	0.1277	19.33	0.0177	2.68
b.....	1.0344	0.2014	19.47	0.0283	2.74

Average dry weight = 19.4 per cent; Nitrogen = 2.71 per cent of moist weight or 13.9 per cent of dry weight.

The acid mixture was boiled 12 hours, filtered and the residue left from the hydrolysis was hydrolyzed again 8 hours in 5 per cent sulphuric acid, and filtered. Neutralized the filtrates separately, made faintly alkaline with sodium hydroxide and then faintly acid with acetic acid. Filtered off the precipitate thus obtained, hydrolyzed this precipitate again separately and united the neutralized filtrate from this to the original solutions. Precipitated the purines from the filtrates separately by the copper sulphate method, obtaining but a small quantity from the second

[1] Bergell and Falk, *Münch. med. Wochenschr.*, lv, p. 2217, 1908.
[2] Higuchi, *Biochem. Zeitschr.*, xvii, p. 21, 1909.

hydrolysis solution, showing that the hydrolysis had been practically complete. United the purine precipitates, decomposed with hydrogen sulphide, filtered, washed thoroughly, and reprecipitated the purines with copper sulphate. After decomposing this second precipitate with hydrogen sulphide the filtrate was made up to 3500 cc. and two 35 cc. samples taken for nitrogen determination. These samples each contained 0.0087 gram N, equivalent to 0.87 gram purine nitrogen in the entire 1530 grams fresh placenta; therefore the purine nitrogen is 2.1 per cent of the total nitrogen. On the basis of about 45 per cent nitrogen in the purines the total amount of purines in this quantity of placenta is approximately 2 grams, corresponding to 0.13 per cent of the fresh weight, or 0.66 per cent of the dry weight. Burian and Schur[1] state that in 100 grams of fresh tissue the following amount of purine nitrogen is present; muscle, 0.06 gram; calves' thymus, 0.45 gram; calves' liver, 0.12 gram; spleen, 0.16 gram. In comparison with these results we find that in 100 grams fresh placenta there is 0.057 gram purine nitrogen, or 0.29 gram in each 100 grams dry tissue.

The purines were then isolated in the usual manner. No uric acid was found. An abundant residue, insoluble in ammonia was obtained, which weighed after repurification 0.62 gram, and which was identified as *guanine*.

A minute quantity of *xanthine*, approximately 0.030 gram, was obtained and identified.

Picric acid precipitated an abundant quantity of typical adenine picrate, weighing 1.5842 grams, which corresponds to 0.586 gram free *adenine*. Melting point 273° (uncorr.).

The purines of the filtrate from the adenine picrate were precipitated with copper sulphate, to free them from picric acid. After washing the copper salt of the purines free from picric acid it was decomposed with hydrogen sulphide, and the purines in the filtrate precipitated with ammoniacal silver chloride. This salt was dissolved in a minimum quantity of boiling dilute nitric acid, filtered hot, and from the filtrate there crystallized out on cooling 0.534 gram typical hypoxanthine silver nitrate, corresponding to 0.2 gram *hypoxanthine*.

To summarize, there were obtained from 1530 grams placenta 0.62 gram guanine, 0.03 gram xanthine, 0.586 gram adenine, and 0.2 gram hypoxanthine, a total of 1.436 grams of purines. This is somewhat below the 2 grams of purines that were to be expected from the quantity of nitrogen present in the purine precipitate, but the discrepancy is probably due largely to the unavoidable losses that occur in the process of isolation and purification of the individual purines. As the evidence obtained from other sources is to the effect that living tissues contain no free xanthine,

[1] *Arch. f. Physiol.*, lxxx, p. 308, 1900.

the presence of a minute quantity of this purine in the placenta may be explained in one of two ways: Either there was a slight degree of autolysis in the placentas during the few hours that elapsed after their expulsion and their immersion in the sulphuric acid, or else the xanthine may have been present as a result of autolysis in the necrotic, infarcted areas which are to be found in all full-term placentas.[1] The presence of so considerable a quantity of hypoxanthine, about one-seventh of the total purines, is in harmony with the general observation that hypoxanthine may occur free in tissues independent of deamidization processes, and may be taken as corroborating the views of Bergell and Falk[2] that the placenta is an organ where active metabolic processes are going on, and not a mere filter which simply regulates the quantity and quality of exchange between maternal and fetal blood.

PURINE ENZYMES OF THE PLACENTA.

In a preceding paper[3] it was shown that the human placenta at full term has no power of destroying uric acid, thus resembling all other human tissues studied. The results obtained by permitting extracts of placenta to act upon uric acid in the presence of a constant current of air are given in the following table:

	Tissue.	Uric acid added.	Uric acid recovered.	Per cent recovered.
1.	Placenta (boiled)	0.1427 gm.	0.1372 gm.	96
2.	Placenta (fresh)	0.1513 "	0.1467 "	97
3.	Placenta (fresh)	0.1486 "	0.1399 "	94

The following experiments demonstrate the presence of adenase and guanase in placenta, and the absence of xantho-oxidase.

Experiment XIII.—980 grams of placental tissue, from two placentas, were ground up, placed in 2200 cc. water with toluol, and autolysis conducted at 37° with a current of air running through the mixture at intervals. After four days added 143 grams more of fresh placenta, to introduce fresh oxidizing enzymes, if such were present, to act upon the purines

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[1] See Williams: *Johns Hopkins Hosp. Bull.*, ix, p. 431, 1900.

[2] Bergell and Falk: *Münch. med. Wochenschr.*, lv, p. 2217, 1908.

[3] Wells and Corper: This *Journal*, vi, p. 321, 1909.

that might have been set free by this time. The autolysis was continued 30 days with intermittent flow of air, and in the filtrate from the coagulated protein was obtained 0.24 gram *xanthine* and 0.265 gram *hypoxanthine* (0.71 gram hypoxanthine silver nitrate).

As no free guanine or adenine was present we have evidence of the presence of both guanase and adenase, while the absence of uric acid indicates the probable absence of xantho-oxidase. It is interesting to observe that the amount of xanthine and hypoxanthine obtained was about equal, corresponding to the approximately equivalent amounts of guanine and adenine found by hydrolysis of the placenta, in contrast with the results obtained by autolysis of fetal tissues which always gave about twice as much xanthine as hypoxanthine.

Experiment XIV.—423 grams fresh placenta were ground, extracted over night at room temperature with 2 volumes of water and strained. To one-half the extract was added 0.1116 gram of xanthine and water to 500 cc. and the mixture allowed to autolyze at 37° with a slow current of air running through it constantly for 48 hours. On analysis at the end of the experiment there was recovered 0.1140 gram of xanthine. There was no uric acid present, not even enough to give a murexide test.

This experiment corroborates the result of the previous experiment in showing the absence of xantho-oxidase. The presence of only adenase and guanase in the mature placenta, as shown by these experiments, does not indicate the probability of any vicarious purine metabolism being performed by the placenta for the fetus, since at this time the fetus is already equipped not only with adenase and guanase, but also with xantho-oxidase.

SUMMARY.

The several enzymes which accomplish purine metabolism in the human organism are developed independently as to time during the course of intrauterine life, the fetus at term being equipped with the same enzymes as the adult, thus differing from the dog and pig.

Guanase is present in the fetus at the third month, the earliest stage studied, and at all later periods.

Adenase is absent in the three-months fetus, but is present at

the fifth month, intermediate stages not having been examined. This demonstrates that these two amidases are not only independent enzymes, but that they are developed independently.

The liver of the human fetus at and after the fifth month contains both adenase and guanase, thus differing from the dog and pig liver.

Xantho-oxidase is demonstrable in the liver, and the combined viscera at full term, but not in the muscle, intestine, kidneys, spleen and thymus. It cannot be demonstrated in the liver or other tissues at or before the sixth month, presumably being developed between that time and full term.

Evidence of the lability of xantho-oxidase is obtained, it being destroyed by long standing, or when tissues other than the one containing the xantho-oxidase are present in an autolyzing mixture.

At no time in intrauterine life, or afterwards, does the human tissue seem to have active uricolytic properties.

Fetal tissues seem to contain much more guanine than adenine, since upon autolysis the resulting solution is constantly found to contain about twice as much xanthine as hypoxanthine.

Human placenta contains about 14 per cent of nitrogen (dry weight) and about 2.1 per cent of this is purine nitrogen; that is, about 0.13 per cent of the fresh weight or 0.66 per cent of the dry weight consists of purines. Of the purines approximately 45 per cent is guanine, 40 per cent adenine, and 15 per cent hypoxanthine. A trace of xanthine may also be found, but the amount is so small that it probably is the result of either post-mortem autolysis or of autolysis in infarcted areas.

The presence of xantho-oxidase or uricase could not be demonstrated in mature placenta.

SOLUBLE CHITIN FROM LIMULUS POLYPHEMUS AND ITS PECULIAR OSMOTIC BEHAVIOR.

By C. L. ALSBERG and C. A. HEDBLOM.

(From the U.S. Bureau of Fisheries Laboratory at Wood's Hole and the Department of Biological Chemistry of the Harvard Medical School.)

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It has been claimed by some investigators that chitin from different organisms varies in some of its properties. Krawkow[1] found that chitin from the spider gives a yellow coloration with iodine which does not change to red with sulphuric acid, while that from *Aphrodite aculeata* gives with iodine an intense violet coloration, turning to red with sulphuric acid. The elementary analyses, however, give closely approximating figures for the forms studied.

It seemed to us that if there were any appreciable variation it ought to be most marked in organisms far removed phylogenetically from the other forms. Since *Limulus*, the horse-shoe crab, is such a form and since it is plentiful about Wood's Hole, we decided to isolate its chitin in order to study its composition and properties. This has already been done by Halliburton,[2] but no analysis was published: a gap we proposed to fill.

INSOLUBLE CHITIN.

The material we used was derived from animals that had been sent to Boston and preserved for nine months in cold storage at a temperature below 0° C. The skeletons, mechanically freed from fleshy parts, were treated for four days with 5 per cent hydrochloric acid; rinsed and heated on the water bath for four hours with 20 per cent potassium hydroxide. The material then gave

[1] *Zeitschr. f. Biol.* xxix, p. 177–185, 1892.

[2] *Proc. Roy. Soc. London*, xxxviii, p. 75–76; *Quarterly Journ. Micr. Science* (n.s.), xxv, pp. 173–181, 1885.

none of the protein color reactions, while the potassium hydroxide solution had turned a dark caramel color. It was next thoroughly washed with distilled water and allowed to stand for an hour in 1 per cent hydrochloric acid, after which the acid was thoroughly washed out. Finally it was boiled out repeatedly with 95 per cent alcohol and treated for half an hour with ether. The resulting preparation was clear, with a slight pinkish tinge. To remove this very slight coloration some of the preparations were treated with warm permanganate of potassium followed by warm dilute sulphuric acid and thorough washing. The material was ground up, a most laborious and unsatisfactory process. The ground material was not very fine and did not appear as homogeneous as one might wish, a fact which is perhaps reflected in the small variations in our analytical results. The preparation was dried *in vacuo* over sulphuric acid at 80° to constant weight. It contained a mere trace of ash. On analysis it gave the following figures:

PREPARATION A.

C *per cent.*	H *per cent.*	N (Combustion acc. Dumas) *per cent.*
47.13	6.50	7.90
47.61	6.58	7.2
47.50	6.65	7.2

Its elementary composition is therefore similar to that of other forms,[1] carbon and nitrogen content being somewhat high, but not more so than in some other animals.[2] Its properties agree essentially with *Limulus* chitin prepared by Halliburton.[3] Like his preparation it is white, insoluble in water, weak acids and concentrated alkali; soluble in concentrated acids, yielding reducing material, presumably glucosamine. Iodine colors it a dark reddish brown. The shade varies a little with the pieces from different parts of the skeleton. The thin lamellar pieces all color alike. while the thick spongy pieces turn a somewhat

[1] Cf. v. Fürth: *Vergleichende chemische Physiologie der niederen Tiere*, p. 472.

[2] Since the presentation of this research, J. C. Irvine, Jr., (of the Chem. Soc. London, Apr., 1909) has shown by polariscopic means that all chitins are probably identical. He also expresses the opinion that the molecular weight of chitin is greater than usually given.

[3] *Loc. cit.*

more violet shade. This is in accord with the observations of Zander.[1] Ferric chloride turns the color to light brown; nitric and sulphuric acids merely slowly decolorize without changing the color. Heating to boiling with water decolorizes. Sodium chloride does not affect the color reaction.

SOLUBLE CHITIN.

In addition to preparation A we made another from skeletons which had been placed in weak hydrocloric acid to decalcify and which, through neglect, had been left in the acid for nine months. The strength of the acid cannot now be given. It was made up at haphazard but was almost certainly not stronger than 5 per cent. The commercial grade of acid was used. From these skeletons, taken directly from the acid without drying, chitin was prepared as above. In the course of this preparation we were astonished to observe some peculiar phenomena. On allowing a part of the purified material to stand for two hours in distilled water, we noticed that a considerable portion of it had gelatinized into large, swollen, transparent lumps and that the water was becoming somewhat thick and opalescent. The solution could be filtered fairly clear through raw silk; and, when evaporated to dryness on the water bath yielded dry brown scales, practically insoluble in cold or hot water. Evidently the chitin had undergone a change which favored its passage into a colloidal solution. The latter, when the water is driven off by evaporation on the water bath, leaves the chitin in a form in which it is not easily soluble again. It would seem, however, that this change, whatever it may be, is not purely a physical one, for such chitin when analyzed before it has been allowed to gelatinize gives the following figures:

PREPARATION B.

C *per cent.*	H *per cent.*	N (Combustion) *per cent.*
45.40	5.14	6.80
45.00	5.44	6.70
45.10	5.47	6.80

A portion of this preparation was decomposed with concentrated

[1] *Arch. f. d. ges. Physiol.*, lxvi, p. 545, 1897.

hydrochloric acid and white crystals obtained, which were recrystallized from 80 per cent alcohol. They were soluble in water; reduced Fehling's solution and gave an osazone melting at 220° (uncorr.). Except for the too high melting point of the osazone, which requires reinvestigation, this substance corresponds to glucosamine hydrochloride.

In attempting to analyze the phenomenon of gelatinization further we endeavored to collect enough "soluble chitin," as we may call our colloidal solutions, to analyze it. The preparation continued to gelatinize slowly on standing in cold water, but it was difficult to obtain enough of the soluble chitin for analysis in this way. Boiling or heating on the water bath for ten days did not materially hasten gelatinization. A portion was superheated in an autoclave for two hours at 1 kilo per sq. cm. pressure. As a result some of the swollen gelatinized portion passed into solution; but the unswollen, ungelatinzied, opaque portion seemed quite unaffected. These opaque areas were present only in the interior of the larger lumps, as though the agent producing the power to gelatinize had not penetrated into the interior. The watery solution was clear and gave the Molisch reaction after concentration. Evidently the whole effect was not great. Thereupon another portion was heated at 6–7 kilos per sq. cm. pressure for three hours. Both the chitin and the watery solution turned brown, the latter giving the Molisch reaction without concentration. The unswollen, opaque, ungelatinized portion, however, seemed unaffected. A third portion was heated in the autoclave for three hours at 2–3 kilos pressure per sq. cm. The filtrate on concentrating became yellowish and a slight precipitate formed. It reduced Fehling's solution slightly; gave no insoluble osazone; but did give a white waxy precipitate when treated with benzoyl chloride according to Schotten-Baumann. Our various efforts to obtain quickly and easily larger quantities of soluble chitin were unavailing. The material gelatinizes easily; but its passage into solution is very slow.

Though we had difficulty in obtaining enough soluble chitin for analysis, it is not because fairly concentrated chitin solutions cannot be obtained. By repeatedly treating gelatinizing chitin in the cold with distilled water; then decanting off, filtering through raw silk, and concentrating the filtrate on the water bath, an

opalescent thick solution was obtained, nearly free from ash, and of about 0.3 per cent concentration. Similarly by first heating to boiling repeatedly and then duplicating the above process a similar thick opalescent solution of 0.6 per cent concentration and practically free from ash was obtained. The concentration was determined by drying down a measured volume of the solution to constant weight and weighing the residue. These thick concentrated solutions showed a certain tendency to gelatinize spontaneously, as though the colloidal solution were reversible. We have not, however, as yet studied the conditions which influence this reversibility. Though soluble chitin is probably reversible as long as it remains in solution, after all the water is driven off by heat and the residue quite dry, it is again as insoluble as the original chitin.

The solution of soluble chitin of 0.6 per cent concentration above referred to was neutral to litmus. It did not reduce Fehling's solution, nor give a color with iodine. The addition of much alcohol to the solution precipitates the chitin but not quite completely. It is still less soluble in alcohol-ether. The action of diastase, invertin, and emulsin upon it was studied. After being subjected to the action of these enzymes for three days at body temperature the solution did not reduce Fehling's solution. There was only a trace of reduction due to the fact that the enzyme solutions used, themselves reduced slightly. When subjected to the action of lipase no acid was formed. The solution inoculated with a pure culture of lactic acid organisms did not turn acid. Inoculated with yeast no gas was evolved.

Soluble chitin filters exceedingly slowly through ordinary filter paper. Before much has passed through, the solution on the filter thickens and partially gelatinizes. Filtration then almost stops. Therefore as indicated above, we were forced to filter the solutions through raw silk, thus obtaining a thick opalescent solution with possibly a slight amount of material in suspension. Being unable to obtain enough chitin filtered through paper, we had to content ourselves with drying down this mixture consisting mainly of soluble chitin with perhaps a little gelatinized chitin in suspension; and then making the following incomplete combustions:

PREPARATION C.

C	H	N
per cent.	*per cent.*	*per cent.*
30.28	5.8	No
30.30	lost	material.

These results, taken together with the figures obtained for preparation B, were so astonishing that we decided to analyze the swollen, clear, gelatinizing lumps which were slowly passing into solution. These gelatinizing lumps turn an intense violet with iodine, changing to light yellow with ferric chloride. The action of nitric acid, sulphuric acid and sodium chloride upon the stained material is the same as for preparation A. On boiling with water gelatinizing lumps are only slowly decolorized after a long time. The clear lumps were dried like the other preparations. As treatment with permanganate to remove the last trace of color prevents subsequent gelatinization, this procedure was omitted; and in consequence the preparation contained a trace of pigment. The following figures were obtained:

PREPARATION D.

C	H	N
per cent.	*per cent.*	*per cent.*
43.4	6.7	5.61
43.9	6.82	5.88
		5.81

This preparation is therefore intermediate between preparations B and C. Prolonged treatment with acid alone seems, therefore, to increase the oxygen content, gelatinization and solution seems to increase it still more.

The question that first arose in connection with the above described phenomena of gelatinization and the changes it produces in the composition of chitin, was whether this behavior was a peculiarity of *Limulus* chitin, or due merely to a partial hydrolysis resulting from the prolonged treatment with acid. To test this point we returned to the skeletons which had not been subjected to the prolonged treatment with acid but had served as the raw material for preparation A, and endeavored to make them too gelatinize. In this we were entirely unsuccessful. After subjecting them to the same method of purification and allowing the resulting preparation to stand in distilled water for ten days

there was not a sign of gelatinization. A part of the preparation was ground up and kept in water for three months without any change. A portion was heated on the water bath with water continuously for six weeks without effect. A final portion was treated in the cold with 5 per cent hydrochloric acid for two and one-half months and then with distilled water without effect.

As these shells had been kept in cold storage so long, it seemed necessary to see whether quite fresh shells could be made to gelatinize. Quite fresh *Limulus* and lobster shells were heated with 5 per cent hydrochloric acid on the water bath and samples removed at intervals. These were tested for gelatinization, always with negative result. The material was then purified in the usual way; but at no stage did it show signs of gelatinization.

We next prepared chitin from the shells which had been treated with acid for nine months, but which differed from the gelatinizing preparations already studied in that after removal from the acid they had been allowed to become air-dry. Purified in the usual way they did not gelatinize. However, if after treatment with alcohol and ether, and permanganate to decolorize, they were boiled with weak sulphuric acid to remove the excess of permanganate, there was a very little gelatinization. A portion stood in 1 per cent hydrochloric acid in the cold; and then began to gelatinize, but not nearly so vigorously as the material which had not been allowed to become dry.

We then treated some of the same material with 5 per cent hydrochloric acid on the water bath for four hours; then washed thoroughly. A sample removed into distilled water gelatinized vigorously. The preparation was next treated with 20 per cent potassium hydrate for three hours. A portion washed free from alkali did not gelatinize. After treatment for one-half hour with 5 per cent hydrochloric acid it gelatinized vigorously. A portion treated with alcohol and ether; then with permanganate; and lastly with dilute sulphuric acid did not gelatinize even after prolonged standing in cold or hot 5 per cent hydrochloric acid. Treatment with permanganate, with sulphuric acid alone, and with dilute nitric acid seems to prevent gelatinization. Treatment with alcohol and ether, while it does not prevent, has a detrimental effect on gelatinization. As

Sollas[1] states that the prolonged action of strong potassium hydrate causes chitin to be soluble in weak acid it seemed possible that we were dealing with the action of this alkali. As stated above, all our preparations from shells which had not remained for a long time in acid were boiled with 20 per cent potassium hydrate. Nevertheless we did not notice any solution when they were subsequently treated with weak acid. However, we made no examination of the acid to see whether it had taken up chitin. All that we can say is that the solubility under our conditions was not sufficient to cause the pieces of chitin to diminish in size enough to attract attention. Some of the shells which had been in acid nine months were boiled with 40 per cent potassium hydrate for six hours. Another portion was heated with 40 per cent potassium hydrate on the water bath for ten hours. In neither case was there a sign of gelatinization. When, however, some of this material was treated on the water-bath with 5 per cent hydrochloric acid for four hours, thoroughly washed, and then transferred into distilled water, it gelatinized vigorously. Another portion washed free from alkali did not gelatinize when brought into distilled water. However, when treated for half an hour with 5 per cent hydrochloric acid it gelatinized vigorously.

From these experiments we were led to conclude that the prolonged treatment with hydrochloric acid is responsible for the formation of "soluble chitin." It is possible that boiling such chitin with strong potassium hydrate facilitates the subsequent gelatinization. The ability to gelatinize is probably not a peculiarity of native *Limulus* chitin; but a property acquired in consequence of the acid treatment. Whether other chitins behave in the same way remains to be investigated. The acid must be allowed to act for many months. In our preparations it had acted for nine months, while even then the interior of the larger lumps had not yet acquired the power to gelatinize. In another preparation mentioned above two and one-half months were insufficient to produce any perceptible change.

If we seek after the nature of the change in the preparations capable of gelatinizing, our analytical figures are the only data from

[1] *Proc. Roy. Soc. London*, series B, lxxix, p. 476.

which we can form an opinion. Unfortunately from lack of material, these are not very complete, and no very certain conclusions can be drawn from them. In general we see that the carbon, hydrogen and nitrogen content diminishes while the oxygen content increases progressively with increasing solubility of our chitin. This is quite consistently so except for the hydrogen content of preparation B. To be consistent with the figures for carbon and nitrogen this should be higher than in preparation D, while actually it is much lower. We are at present at a loss to account for this observation. Taking the figures as a whole they can most easily be explained by assuming that the chitin has taken up water. Is this merely the water taken up in hydrolysis? In that case our analytical figures must approach as a limit monoacetyl-chitosamin,[1] $C_7H_{15}NO_6$, which has the percentage composition, C, 40.19 per cent; H, 7.1 per cent; N, 6.7 per cent; O, 45.9 per cent. Our preparation D, the gelatinizing lumps, has the average composition C, 43.65 per cent; H, 6.76 per cent; N, 5.77 per cent; O, 43.82 per cent. A comparison of the two sets of figures rather confirms the idea that the process is mainly one of hydrolysis. However, the figures for our preparation, particularly the low H and N values, are not quite what one would expect in an hydrolysis pure and simple. If furthermore we assume that our incomplete data on preparation C, the soluble chitin, shall prove to be correct, it is evident that more has taken place than a simple hydrolysis. Water must, in that case, have been taken up otherwise than by hydrolysis. Moreover a simple hydrolysis which had changed the composition as much as in preparation D would most probably have proceeded until the molecular weight was nearly as low as that of monoacetyl-chitosamin. This, as will be shown, is by no means the case. Of course it does not necessarily follow, because our solutions are colloidal and, as will be shown, do not depress the freezing point markedly, that the dissolved substance has therefore a complex structure. Simple substances, as is well known, may pass into the colloidal state; or even be colloidal in one solvent and not in another, as for instance tannic acid. Still in our case this does not seem very probable. Furthermore, had the hydrolysis

[1] Fränkel and Kelley: *Sitzungsbericht d. k. Akad. in Wien*, 1901, p. 100.

proceeded so far, one would hardly expect soluble chitin to behave like a reversible colloid. We are, therefore, for the present, inclined to believe that the addition of water is a more important factor in the changes that the chitin has undergone in passing into solution, than simple hydrolysis. These changes begin before gelatinization, for even in preparation B we have a low carbon, hydrogen and nitrogen content. It is possible, of course, that we might be dealing with an asymmetrical hydrolysis splitting the molecule up into a number of fragments of varying size one or more of which may be simple bodies. The latter must then be lost in our method of purification, and must leave behind a large complex, very rich in oxygen. The simple bodies, granted they be formed, one would expect to find in the acid in which the shells have been decalcified for nine months. Unfortunately this acid was thrown away before we realized that it had produced changes in the shells. We have looked for the cleavage of acetic acid during the process of gelatinization in distilled water. We failed to find it. As we were compelled to work with small amounts of material much importance must not be attached to our failure. Still another possibility has to be considered. It seems possible that our analytical results are dependent upon the absorption or mechanical retention or imperfect drying of our preparations. We therefore took a sample of preparation D after it was dried to constant weight ready for analysis and dried it for several weeks longer at 80° C. *in vacuo* over sulphuric acid. It showed practically no loss of weight. Evidently insufficient drying is not responsible for our analytical figures. That our method of drying is at least as efficient as the one commonly used is shown by the fact that our carbon values for preparation A are high. If our preparations contained water it does not seem probable that it was in the form of "moisture" as this term is commonly used. The most probable explanation for the present, seems to be that our "soluble chitin" contains water in firm combination.[1] It

[1] An opinion as to the capacity of chitin to take up water quite in harmony with this view was expressed many years ago by Sundwik (*Zeitschr. f. physiol. Chem.*, v, p. 393): "Eine solche Wasserabspaltung ist nun unter den mehrwerthigen Alkoholen nicht ungewöhnlich, und dass im Chitine das Wasser beim Trocknen sehr hartnäckig zurückgehalten wird, spricht dafür, dass dasselbe nicht einfach durch seine Hygroscopicität,

does not seem probable that the formation of our "soluble chitin" is merely a depolymerization, or even a simple hydrolysis; but that perhaps in addition to these processes water takes a part in some other way than hydrolysis. If this view be correct, we may have here a case in which a substance in passing into the colloidal state combines with the medium in which it dissolves, perhaps undergoing in addition a certain amount of hydrolysis. In accord with this view is the fact that the process seems to some extent reversible. We had reached this conclusion some time ago.[1] Since then Fouard,[2] who for some time past has been making most interesting studies upon soluble starch, has published similar conclusions. If these views shall be substantiated and shall prove more than a special case, this phenomenon may turn out to be of considerable biological interest. We believe it furnishes the key to an explanation of the peculiar osmotic behavior of soluble chitin as described below.

THE MOLECULAR WEIGHT OF CHITIN.

All investigators who have hitherto studied the cleavage of chitin were unable to decompose it without breaking its molecule into rather small fragments. It is possible to dissolve chitin in strong acids, and it takes some little time before it is decomposed by them. To get it into solution by any other less drastic means has not, hitherto, been possible. We have, as we believe, for the first time put it in solution in other than concentrated acid. Our soluble chitin is probably the least changed derivative or mixture of derivatives hitherto obtained. The fact that it is now possible to obtain chitin in solution will make it easier to investigate its structure. It will be easier to deal with a substance in solution than with an insoluble substance which can only be made to dissolve by dismembering it thoroughly. We

sondern vielmehr durch chemische Vereinigung fest zurückgehalten wird. Ledderhose, der bei 110° C. das Chitin trocknete, hat auch einen viel geringeren Kohlenstoffgehalt im Chitine gefunden." Sundwik dried his preparations at 132°–135° C.

[1] *Proc. Amer. Soc. of Biol. Chemists*, i, p. 192.

[2] *Compt. rend. de l'Acad. d. Science*, cxlviii. p. 502. *Zeitschr. f. Chem. u. Industr. d. Kolloide*, iv, p. 185.

have already taken advantage of this opportunity to make some studies upon the probable molecular weight of chitin. There is in the literature much difference of opinion as to the size of the molecule. Araki[1] gives the formula $C_{18}H_{30}N_2O_{12}$ (mol. wt. 418). Staedler[2] calculates it $C_9H_{15}NO_6$ or $C_{17}H_{28}N_2O_{11}$ (mol. wt. 436); Ledderhose[3] $C_{15}H_{26}N_2O_{10}$ (mol. wt. 394); von Fürth[4] $C_{18}H_{30}N_2O_{12}$ (mol. wt. 466). Sundwik[5] calculates $C_{60}H_{100}N_8O_{38} + n(H_2O)$ with a value for n of 1 to 4 (mol. wt. 1588). Fränkel and Kelly[6] from a consideration of its cleavage products infer that the molecular weight must be higher than here expressed. Von Fürth and Rosso[7] also state that it is higher. We therefore decided to determine the freezing point of our soluble chitin solutions. We are perfectly well aware that such determinations upon colloidal solutions have very little value and that no very definite conclusions can be based upon the values obtained.[8] However, these cryoscopic determinations must show one thing, viz: that the molecular weight of the substance under investigation, in the state under consideration cannot be less than is indicated by the lowering of the freezing point. It may, however, be much greater. It was from these considerations that we undertook our determinations. We expected to learn not the actual molecular weight; but to learn that this weight is greater than a certain minimal value. This in itself, considering the discrepancy of opinion in the literature, ought to prove of interest until the true molecular weight of chitin shall at some future date be established.[9] The material was prepared by heating gelatinizing pieces, as described above; concentrating; dialyzing to remove traces of salts; and finally filtering through silk. During dialysis some of the material passed through the parchment paper membrane. For this reason it was impossible to determine the osmotic pres-

[1] *Zeitschr. f. physiol. Chem.*, xx, 498–500, 1895.

[2] *Ann. der. Chem. u. Pharm.*, 111, p. 31–38, 1859.

[3] *Ber. d. deutsch. chem. Gesellsch.*, ix, p. 1200–1201, 1876.

[4] *Beitr. z. chem. Physiol. u. Path.*, p. 100–114, 1907.

[5] *Loc. cit.*, p. 592.

[6] *Loc. cit.*

[7] *Beitr. z. chem. Physiol. u. Path.*, viii, p. 163, 1906.

[8] Cf. Jacques Duclaux: Cryoscopi des colloides, *Compt. rend. de l'Acad.*, cxlviii, p. 714.

[9] Cf. v. Fürth, *loc. cit.*, p. 480.

sure directly. The finished solution was of 0.7 per cent concentration, and left no ash. The determination of the freezing point gave the following values:

$\Delta = .0100$
$\Delta = .0075$
$\Delta = .0050$
$\Delta = .0075$
$\Delta = .0050$
Average $\Delta = .0070$
Molecular weight = 1974

We are perfectly well aware that such thermometer readings as these have very little absolute value; but nevertheless it is safe to conclude that the molecular weight of chitin in this particular colloidal solution is at least five times as great as that generally given. Leaving aside the faults inherent in the method, already discussed, we must further consider that this material is, as suggested, a cleavage product, or a mixture of cleavage products of insoluble chitin. It is reasonable to assume, therefore, the molecular weight of chitin to be much greater than 2000; or perhaps it is safer to say that it is probably of the same order of magnitude as that of soluble starch, for there is at present a tendency to regard the molecular weight of the latter as much smaller than formerly assumed[1]

THE OSMOTIC BEHAVIOR OF SOLUBLE CHITIN.

In the course of our work in preparation of soluble chitin for freezing point determinations, we observed some very puzzling phenomena. A soluble chitin solution prepared in the cold was placed in a Schleicher and Schüll diffusion capsule ("Diffusionshülse") The filled capsule was placed in a beaker of distilled water. Three days later the capsule was both empty, save for a slight trace of gelatinized material in the bottom, and floating on the top of the liquid. A second capsule, carefully tested for a leak, behaved similarly. In both cases the distilled water on the outside had become opalescent and contained soluble chitin. The capsules did not rise to the surface because they

[1] Cf. Cross and Bevan: *Ber. d. deutsch. chem. Ges.*, xlii, p. 2198.

were lighter than the liquid, for if a capsule be torn open it sinks. The conditions of the experiment were then reversed. The distilled water was put inside the capsule and the chitin solution on the outside. This time the level of the liquid rose inside the capsule. This capsule was then washed, refilled with 2 per cent maltose solution, and placed in distilled water. It behaved in the familiar way, the level at first rising inside the capsule. The capsule was then emptied of its maltose, refilled with chitin solution, and placed in water. Again it behaved like the first two capsules. It was thought possible that this behavior was peculiar to these capsules. Therefore collodion and parchment paper membranes were tried a large number of times. They behaved in exactly the same way except that the phenomenon was slower for the collodion membrane. Some of the experiments were carried out at a temperature but slightly above 0°, to avoid bacterial action. No different results were obtained.

At present we are not ready to offer an explanation for these most peculiar osmotic phenomena by which the dissolved substance passes through the membrane together with the solvent. We have devised an hypothetical explanation, and are testing it experimentally. Our reason for publishing at this time the bald facts is that it will be a long time before we can finish our work upon these osmotic phenomena. It takes many months to prepare soluble chitin; and collaboration is more difficult because one of us has changed his residence. This is also the explanation for the incompleteness of much of the rest of the paper.

SUMMARY.

Limulus chitin has in general the same percentage composition and properties as that from other animals.

By very prolonged treatment with weak hydrochloric acid in the cold it acquires the power first to gelatinize and then to form a colloidal solution in water. Such solutions do not reduce alkaline copper solutions; and are not colored by iodine. The chitin after the prolonged treatment with hydrochloric acid does not gelatinize when in the process of purification it is treated with strong potassium hydrate. It cannot be made to gelatinize until after the removal of the potassium hydrate with hydrochloric acid.

The analytical figures obtained for preparations of soluble chitin and gelatinized chitin are best explained by the hypothesis that chitin in passing into colloidal solution combines with solvent water, and is perhaps also in addition hydrolyzed. The solvent takes part in the process.

Soluble chitin depresses the freezing point but slightly, so that its molecular weight is probably very great.

Soluble chitin dialyzes and has the peculiar property of carrying the water in which it is dissolved through the membrane, so that the space within in the latter may become nearly empty.

SOME OBSERVATIONS ON THE STUDY OF THE INTESTINAL BACTERIA.

By ARTHUR I. KENDALL.

(*From the Laboratory of Dr. C. A. Herter, New York.*)

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The alimentary canal may be regarded from the point of view of bacterial processes within it, as a singularly perfect incubator; an incubator in which there is provided at different levels such a range of reaction and diversity of food that not only are the conditions suitable for the growth of the normal habituated intestinal bacteria but often also for those organisms, capable of developing at body temperature, which are ingested with the food of the host.

An idea of the truly enormous daily bacterial proliferation which takes place in the intestinal tract may be obtained if one remembers that a considerable portion of the fecal mass is made up of the bodies of bacteria, dead and living. At the same time the multiplicity of types and variety of physiological requirements of this intestinal flora are indications of the excellence of the incubator and a strong reminder of the influence which the unrestrained activity of these organisms might conceivably exercise upon the general condition of the host.

The possibilities of bacterial invasion through the intestinal portal of entry have not been overlooked by investigators, and, indeed, among the most brilliant chapters of medicine are those concerning the etiological relationships which have been demonstrated between certain pathogenic bacteria of exogenous origin and specific diseases of the intestinal tract, for example, typhoid, cholera and dysentery.

The very importance of these discoveries has been a potent factor in diverting attention from the studies of the normal intestinal flora with its wealth of problems relating to the principles which govern the activity of these bacteria. Even at the present time the sequence of events which permits the establishment of

these exogenous invaders in the alimentary canal and the exact conditions through which they are able not only to extend and maintain themselves but even to replace wholly or in part the normal flora, are unknown.

It is possible to trace the influence of these epoch-marking studies in the subsequent history and development of Intestinal Bacteriology.

It appears to be a fact that the majority of bacteria of exogenous origin, pathogenic for man (excluding the anaërobes) are relatively inert from the standpoint of chemical activity. On the other hand, these organisms grow in more or less distinctive ways in artificial media, and, usually, they may be recognized by their cultural aspect, their inability to bring about deep-seated changes in their nutrient environment, through specific serum reactions or by their power to initiate characteristic lesions in susceptible animals. In these respects these exogenous organisms contrast in a noteworthy manner with many prominent types of the normal intestinal bacteria.

The more prominent of the latter are distinguished by their chemical or physiological activity and their identification depends far more upon their ability to bring about well-marked chemical changes in their nutrient environment than upon their cultural properties or serum reactions.

The lack of appreciation of this fundamental difference which exists between the relatively inert pathogens and the chemical activity of the more important types of the normal intestinal flora, together with the notoriety that attaches to the former, explains the unprogressive attitude which has characterized many researches on intestinal bacteriology.

While it must be admitted that the purely academic methods of research have resulted in scores of more or less complete morphological and cultural descriptions of bacteria of intestinal origin this knowledge is fragmentary and unclassified. It is devoid of data which would permit one to correlate the presence of these organisms with the diet or condition of the host, or even to form a judgment concerning their numerical relations with other intestinal organisms.

This "bacteriocentric" conception is not illogical when one is dealing with the exogenous pathogens mentioned above, but

it is unproductive of definite results when it is applied in its unmodified form to the study of the normal intestinal flora. It is becoming more and more evident that the problem of intestinal bacteriology must be approached from the dynamical rather than from the cultural standpoint.

Dr. Theobald Smith[1] has stated the case admirably in the following terms: "It is what bacteria do rather than what they are that commands attention, since our interest centers in the host rather than in the parasite."

It is the purpose of this paper (having called attention to the inadequacy of purely academic methods) to indicate in a general way the procedures and use of media through which one may obtain a more comprehensive idea of the significance of bacterial activity in the intestinal tract. For the sake of simplicity, it will be assumed that the host is an experimental animal (preferably a monkey, since its physiology more nearly approaches that of man) under absolute control. Its diet can be regulated at will and its excretions, particularly the urine and feces, can be collected in an uncontaminated state. The diet of this animal may be either purely protein in nature (e. g., hard boiled eggs) or may be carbohydrate. For the latter it has been found that milk with some added dextrose is excellent. This combination contains considerable protein, but, as has been shown in a previous communication,[2] the flora developed is acidophilic and not proteolytic in nature. It should be stated parenthetically that a diet consisting wholly of carbohydrate would be less suited for bacterial development since bacteria need some nitrogen in their food.

The host (monkey) is fed daily with protein or carbohydrate, as outlined above. As this food passes through the alimentary canal from mouth to anus, it is subjected to the action of ferments elaborated by the host. Also, at different levels of the tract it is decomposed in part by various types of bacteria. The predominating types of bacteria which take part in this decomposition are determined largely by the nature of the diet.

When a change is made in the animal's diet from protein to

[1] Theobald Smith: Some Problems in the Life-history of Pathogenic Microörganisms, *Amer. Med.*, viii, pp. 711-718, 1904.

[2] Kendall: This *Journal*, vi, pp. 257–269, 1909.

carbohydrate, or the reverse, it would seem at first sight that two possibilities exist with respect to the behavior of the bacterial flora towards these alterations in pabulum. First it would appear that the types represented might undergo relatively little change, owing to the fact that they accommodate their metabolism to either form of diet; or, secondly it is conceivable that there might be a shifting of the dominant organisms so that upon the protein diet the proteolytic bacteria will be prominent, while acidophilic bacteria[1] will become dominant as the carbohydrate is increased.

Previous experiments[2] have shown that the latter possibility is the one most commonly realized, namely that there is a parallelism between the nature of the diet and the character of the bacterial types represented in the intestinal and fecal flora. Definite evidences of this activity of the intestinal flora are not wanting. In the excretions (particularly in the urine) there occur substances which are the products of bacterial metabolism. These end products of bacterial digestion may be burned in the body, excreted direct, or combined with some substance or substances elaborated by the host to render them less toxic and excreted in this combined form. The presence of these metabolic products in the urine is influenced by two principal factors—bacterial activity and intestinal absorption. Other considerations enter into the problem, and for this reason the qualitative, rather than the quantitative estimation is all that is to be considered in this connection. It follows that the recognition of these end products, *which are in reality indicators of certain definite types of bacterial activity*, is of the greatest importance. Hitherto this correlation between diet, intestinal flora and end products has been largely overlooked, and the natural result has been that the corroborative evidence which these indicators furnish has not been utilized.[3]

[1] For a discussion of the bacterial changes associated with a change in diet see Kendall: This *Journal*, vi, pp. 266–268, 1909.

[2] See Kendall: *loc. cit.*

[3] It is undoubtedly true that some connection between diet and bacteria on the one hand, bacteria and end products on the other, has been surmised, but the lack of definite information available at the present time is strong evidence of the truth of the assertion that the *three* phases—diet, bacteria and end products—have not been considered in their interdependent relations.

Having determined by experiment that a given diet (for example, simple protein) is associated with a definite type of bacterial activity, and that coincidentally certain of these indicators are present in the urine of the host, it becomes a relatively simple matter to isolate individual strains of this fecal flora which will reproduce, either alone or symbiotically with other strains, these same end products. This is accomplished by growing the mixed fecal bacteria in media of the *same fundamental composition* as that of the diet which originally nourished them. An enrichment of the dominating types usually takes place, the abrupt change from intestine to media, with its resulting lack of development is partially overcome, while every possible opportunity is given for the selective development of the desired types. Thus the plating, which must be relied upon for the final separation of the cultures in a state of purity, is far more successful than when plating direct, without the preliminary enrichment, is resorted to. These end products, then, become the criteria through which it is possible to decide with definiteness the participation, indifference or antagonism of each of these types of bacteria in the process under consideration.

In the present undeveloped state of the subject it will be impossible to formulate a definite procedure applicable to all cases. It is very probable, indeed, that from the nature of the phenomena involved, such an undertaking would be disappointing in its results. The best that can be done will be to outline the course of a definite experiment, indicating the procedures through which it is possible to arrive at the desired conclusions.

Before this is done, however, I wish to mention briefly those developments and extensions of present methods which have made it possible to bring the work to its present state. They are: the association of certain products of bacterial metabolism (which are present under specific conditions in the urine of the host) with the activity of certain definite types of organisms upon definite foodstuffs; the corroborative use of artificial media for the demonstration of the completeness and direction with which the bacterial complex in the intestinal tract follows the changes in the character of the diet, and the employment of these media for the selective enrichment and isolation of those varieties of organisms which are most intimately concerned in these changes and the elaboration of these end products.

The following experiment which was repeated several times, always with the same results, may be quoted to demonstrate the general procedure followed in this work.

A monkey was placed upon a diet consisting of milk plus dextrose. Bacterially considered, this diet was essentially carbohydrate in character—there were very few proteolytic bacteria present in the fecal flora, which was of the acidophilic type; the fecal bacteria which developed on this regimen grew less readily in artificial media than was the case with either a mixed or a protein diet.

The acidophilic nature of the fecal flora was brought out in a striking manner by inoculating with the mixed fecal flora milk fermentation tubes,[1] broth fermentation tubes containing dextrose, lactose and saccharose, gelatin and a series of dextrose broth containing varying amounts of acetic acid.[2]

The milk tubes showed coagulation, but no further action was apparent. In the broth fermentation tubes there were slight turbidities with very little or no gas. The gelatin tubes contained only a very slight growth after many days, while the acid dextrose tubes showed moderate development, even in the highest acidities. The milk and gelatin are particularly noteworthy. Milk, and to a lesser extent, gelatin, are excellent media for the development of proteolytic bacteria, while the acidophilic flora grow much less readily on artificial media than do the protein bacteria. Hence the lack of bacterial development in these media is the strongest evidence of the inhibition, or *even replacement of proteolytic organisms* by the acidophilic flora.

The urine was found to be free from indican, phenolic bodies and other products of intestinal putrefaction.

The animal was then placed upon a purely protein regimen with an ample allowance of water.

The conditions changed rapidly. The milk fermentation tubes became the seats of great bacterial activity when they were inoculated with the mixed fecal flora. The milk was greatly peptonized and much gas was formed; liquefaction was marked in the gelatin tubes (stab inoculations); the fermenta-

[1] Theobald Smith, Herbert R. Brown and Ernest L. Walker: *Jour. Med. Research*, xiv, pp. 193–206, 1905.

[2] Hayem's solution. See Finkenstein: *Deutsch. med. Woch.*, p. 263, 1900.

tion tubes showed large amounts of gas (even 90 and 100 per cent being not infrequently produced in eighteen hours); while there was a gradual diminution in the acidophilic flora grown in acid broth tubes. This diminution was manifested chiefly by the inability of the organisms to grow in the highest acidities. These growths took place very rapidly, eighteen to twenty hours being ample time for the described phenomena to develop in their completeness.

Coincidentally products of the decomposition of protein began to appear in the urine. Indican and phenolic bodies were particularly sought for and found in increasing amount as the proteolytic flora became established. Urorosein was not found.

The replacement of the acidophilic flora, then, was demonstrable in the following manner:

a. There was a *microscopical* change in the fecal flora. The strongly Gram-positive fields, consisting largely of the medium sized rod-shaped, acidophilic organisms were replaced by large, Gram-positive rods; smaller, Gram-positive and Gram-negative rods [subtiloid bacilli]: coccal forms in small numbers, and oval, Gram-negative bacilli, referable morphologically to *B. coli* and related aërogenic bacilli.

b. Culturally, whereas the acidophilic (carbohydrate) flora grew very poorly or not at all in gelatin; slowly, with at most coagulation, in milk; moderately, with little or no gas in fermentation media; and considerably in even the highest acidities ($\frac{N}{10}$) in dextrose broth, the exact reverse was the distinguishing feature of the protein diet. Gelatin was promptly liquefied; peptonization and considerable gas-production were features of the milk tubes; heavy turbidities and large volumes of gas were produced in dextrose, lactose and saccharose, while the higher acidities of the acid dextrose bouillon cultures were devoid of growth.

c. Chemically, on a carbohydrate diet, with the resulting acidophilic flora, the urine was free from products of intestinal putrefaction. As the protein regimen was established and the proteolytic bacteria became habituated to the changed conditions in the intestinal tract, indican and phenolic bodies gradually became prominent in the urine.

It will therefore be seen that through the use of this general procedure it is possible to demonstrate perfectly definite, con-

sistent correlations between the nature of the diet, the morphology, cultural and physiological relations of the intestinal flora, and the type of and products of bacterial metabolism on each of these diets. These relations are distinctive and sharply defined.[1]

SUMMARY.

The procedures in this paper are outlines of general principles applicable to the determination of the more important types of bacterial activity in the intestinal tract and for the isolation of the principal agents concerned in these processes, rather than specific methods to meet special cases. An extension of these principles, along appropriate lines, however, will furnish a definite line of approach to the study of the majority of problems relating to the intestinal flora.

These procedures are based upon the correlation which exists between diet, bacterial flora and end products of bacterial activity which appear in the urine. The nature of the diet practically determines the dominant types of intestinal bacteria, and these organisms in turn, acting upon the digestive products of the diet elaborate the end products of their activity which appear in the urine.

With the exception of a few anaërobes (which derive their oxygen from the combustion of carbohydrates) the majority of the prominent types of the normal flora which develop on a protein diet grow luxuriantly in media free from carbohydrate, while those developing on a carbohydrate regimen grow poorly, or even not at all, unless carbohydrate is present. Hence by inoculating portions of the mixed fecal flora with gelatin and milk and observing the degree and rapidity of peptonization, it is possible to form a judgment of the character of the proteolytic flora. At the same time these media furnish conditions so favorable for the growth of these organisms that they can be regarded as selective for the isolation of the proteolytic flora.

On the other hand, through the use of media containing carbohydrate and particularly the acid dextrose broth, one obtains a

[1] A detailed account of these experiments carried out on monkeys, using these procedures, will be published later.

fairly specific enrichment of the acidophilic flora, characteristic of a carbohydrate regimen.

Furthermore, through the use of these selective media it is possible to form a judgment of the completeness of the bacterial response to the nature of the diet. For example, if the experimental animal is on a carbohydrate regimen, the presence or absence of growth in protein media will indicate the presence or absence of proteolytic bacteria, since the acidophilic organisms do not grow well in these media and cannot, therefore, inhibit the growth of these organisms. Conversely, with a protein diet, the presence or absence of acidophiles may be determined by inoculating the mixed fecal flora into acid dextrose broth, which is unfavorable for the development of the proteolytic types. These determinations may be made roughly quantitative for the different types by inoculating definite amounts of the mixed fecal flora into appropriate media.

The end products of bacterial activity which appear in the urine are important for two reasons: they indicate the types of bacterial activity in the intestinal tract, and their reproduction in artificial media by pure cultures derived from the intestinal flora furnish strong presumptive evidence of the participation of these organisms in the process.

A STUDY OF THE CHEMISTRY OF BACTERIAL CELLULAR PROTEINS.

By SYBIL MAY WHEELER.

(*From the Hygienic Laboratory of the University of Michigan.*)

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It is the object of this paper to present the results of a study of some of the bacterial cellular proteins from a chemical standpoint. The design has been primarily an investigation of cellular chemistry, especially cellular proteins, but for purposes of comparison other proteins, both animal and vegetable in origin, have been studied. The writer has given special attention to the poisonous group of the proteins though this has been approached by varied methods. After speaking briefly of the preparation of the cellular proteins and of their general reactions, digestion with pepsin and trypsin, the effect of cleavage with dilute mineral acids, and with concentrated acids will be discussed; then the cleavage with dilute alkali, in both water and alcoholic solution, which separates the poisonous from the non-poisonous portion, will be taken up. With only a brief statement of the properties of the non-poisonous portion which has been more closely studied by Leach and Agnew in this laboratory, the chemistry and cleavage of the poisonous part will be presented.

Although much has been done by many investigators upon the varied phases of the chemistry of bacteria little has a direct bearing upon the work here presented, hence for the sake of brevity a résumé of the literature will be omitted at this time.

Preparation of Material.

Both pathogenic and non-pathogenic organisms have been grown and studied. Among these may be mentioned the typhoid

bacillus, colon, anthrax, tubercle, timothy, pyocyaneus, proteus vulgaris, subtilis, ruber of Kiel, megaterium, and sarcina lutea. Other proteins used for comparison have been egg albumen, serum albumen, casein, gelatin, Witte's peptone, Defresne's peptone, De Chapoteaut's peptone, Macquaire's peptone, edestin from hemp seed, zein from maize, also the tissue from two cancers. The bacterial cells in all instances, except the tubercle and timothy bacilli, which were grown on the surface of glycerine beef tea in liter flasks and the anthrax bacillus which was grown on agar in Roux flasks, were grown on 3 per cent agar at room temperature in the large incubating tanks in use in this laboratory. The colon and typhoid need to grow for from ten to fourteen days, but the best growth of the non-pathogenic organisms was obtained in about a week. The thick, slimy, bacterial growth was loosened from the subjacent agar with glass rods, bent twice to form rakes, and then drawn into large flasks by means of suction, from which it was poured into large precipitating jars and thoroughly washed by decantation with 95 per cent alcohol. It was next covered with absolute alcohol and allowed to remain for several hours with frequent stirring, when it was filtered and extracted thoroughly with ether in Soxhlets, after which it was dried and ground in a mortar to a fine powder. The anthrax bacilli underwent similar treatment after removal from the agar of the Roux flasks. With the tubercle and timothy organisms the most abundant growth was obtained at the end of a month. To harvest, the beef tea was decanted through a rather hard filter paper leaving the thick heavy scum of germ growth in the flask, into which absolute alcohol was poured and allowed to remain for some time with frequent shaking to break up the bacterial mass and to thoroughly wash it with alcohol. The whole was next poured onto a hardened filter, and after the alcohol had drained off was placed in Soxhlets, thoroughly extracted with ether, often for several days, or until the ether remained clear, dried and ground to a fine powder. The albumen used was obtained by pouring the whites from twenty dozens eggs into alcohol and after carefully washing with alcohol, extracting thoroughly with ether, drying and pulverizing. The casein and serum albumen were preparations of Kahlbaum and the names of the four peptones indicate their source. The gela-

tin, that used in the laboratory for media, was cut into fine bits and thoroughly extracted with ether in Soxhlets. The edestin was prepared from hemp seed by Leach and Bowles in this laboratory. The ground seed, from which the oil had been expressed, was thoroughly extracted in Soxhlets with either benzene or petroleum ether, and the seeds, freed from extractives, were ground to a powder. From this the edestin was obtained by extracting with a hot 5 per cent solution of sodium chloride. The solution was filtered hot and from it on cooling, the edestin settled out, was filtered, washed and obtained as a fine white powder on grinding. The zein was prepared from corn meal by Leach by extracting with dilute alcohol, precipitating with water, redissolving in alcohol, precipitating with ether, washing, drying and grinding to a powder. The tissue from two cancers, removed by J. W. Vaughan, was cut fine, thoroughly extracted first with alcohol, then with ether, both in Soxhlets, after which each was ground to a powder so far as possible. In these ways all the different materials were obtained in the form of a fine powder varying in color from white to a light brown.

The first work here presented was for the most part with the bacterial cellular substances alone, the comparative work with the other proteins being discussed later in connection with the alkali-alcohol extraction of the poisonous group.

General Properties of the Bacterial Proteins.

The alcohol and ether extractions of the germ substances removed in each case considerable quantities of fats and waxes but these have not been studied to any extent other than to determine that these extracts contained no substance poisonous to animals. The bacterial cellular substance freed from all extractives and in the form of a fine powder as mentioned above was insoluble to any appreciable extent in water or other physical solvents, it being necessary to extract repeatedly with heat as shown by Leach (1) first with dilute sulphuric acid (1 per cent to 5 per cent), then with a solution of sodium hydrate (2 per cent to 4 per cent), to bring it into solution. Physical solvents failed to remove any poisonous body, e. g., a physiological salt solution extract of the colon germ showed a small per cent of

nitrogen but no poision, likewise while water and physiological salt solution extracts of the powdered egg albumen showed faint Millon and biuret reactions with a fair Molisch test, they contained no poison as such but instead sensitized the animals to which they were given.

As was shown by Vaughan and Cooley some years ago the poison contained within the cell of the colon bacillus resists a relatively high temperature. Two hundred milligrams of this substance in 10 cc. of water was put into a tube, sealed and heated to from 180° to 184° for half an hour. This treatment was without appreciable effect on the toxicity. After being heated with water at the temperature of the water-bath for one hour, the colon bacillus retained its form, still took the usual anilin stains, though not quite so deeply and lost none of its toxicity. When thus heated with water the cellular protein does not go into solution and the filtrate from a porcelain filter is inert. The same is true of filtered cultures of the living bacillus, even those twenty days old or older. It is evident that this organism does not produce a soluble poison and that its toxic constituent is intracellular. Heating for from one to two hours in an autoclave at 154° and under two kilos of pressure failed to extract the poison from the bacterial cell. In fact the filtrate from a preparation, heated as just stated, and passed through porcelain gave no cloudiness when dropped into absolute alcohol.

Each of the bacterial cellular substances responded readily to all the protein color reactions including the Molisch carbohydrate test and also reducing Fehling solution after boiling with dilute mineral acid.

In order to compare their relative composition, determinations of ash and of nitrogen by the Kjeldahl method were made as follows, the figures given being the average of two or more determinations.

TABLE 1.

Determinations of Nitrogen and Ash in Germ Substances.

Germ Substance.	Per Cent N.	Per Cent Ash.
Typhoid	11.55	5.7
Colon (Leach) (2)	10.65	8.615
Colon (Agnew) (3)		8.38
		7.20 (air dried)
Tuberculosis (Agnew) (3)	10.55	11.47
	9.27 (air dried)	9.98 (air dried)
Anthrax	10.285	7.76
Pneumococcus	10.406	5.78
Subtilis	5.964	10.83
Proteus vulgaris	6.791	10.88
Ruber of Kiel	10.655	6.71
Megaterium	8.349	10.18
Pyocyaneus	10.843	9.04
Violaceus	11.765	6.90
Sarcina aurantiaca	11.460	6.40

As will be seen the nitrogen varied from 5.964 per cent to 11.765 per cent while the ash showed from 5.7 per cent to 11.47 per cent.

Notwithstanding the fact that preparation of the bacterial cellular substances in the form of fine powders as described took away the life of the germs by the thorough extraction with alcohol and ether, the cell still maintained intact its structure under the micr scope and still took anilin dyes perfectly though the tubercle bacillus and the timothy were no longer acid fast. But although the organisms were no longer capable of growth they were all, pathogenic and non-pathogenic, highly poisonous when injected into experimental animals indicating the presence of an intracellular poison. Indeed many of the non-pathogenic germ substances were more poisonous, weight for weight, than that of the pathogenic cells, pointing to the conclusion, suggested by Vaughan, that whether a given bacterium is pathogenic or not depends, not upon the presence or absence of a poison within its complex, but upon whether or not it is able to grow and multiply in the body and likewise to be split up so that its poison is liberated. As the free poison split from the cell will be discussed later the relative toxicity in round numbers of some of the dry, freshly prepared, finely powdered cellular substances,

when injected intraperitoneally into guinea pigs, as determined in this laboratory, may be of interest. The cellular substance is fatal in from 6 to 8 or 10 hours.

TABLE 2.

Relative Toxicity of Dry, Dead Bacterial Cells.

Name of Organism.	Proportion of Cell Substance to Body Weight.
Bacillus anthracis (J. W. Vaughan) (6)	1 : 1,700
Sarcina lutea (Detweiler) (4)	1 : 2,030
Bacillus tuberculosis (Wheeler)	1 : 3,000
Sarcina aurantiaca (Detweiler) (4)	1 : 25,500
Bacillus violaceus (Detweiler) (4)	1 : 26,500
Bacillus megaterium (Wheeler)	1 : 31,000
Bacillus typhosus (Wheeler)	1 : 40,000
Bacillus pyocyaneus (Wheeler)	1 : 50,000
Bacillus coli communis (Marshall and Gelston) (5)	1 : 75,000
Bacillus ruber of Kiel (Wheeler)	1 : 77,000
Bacillus prodigiosus (Detweiler) (4)	1 : 90,000

Notwithstanding the fact that the bacterial substances are so poisonous when injected intraperitoneally and that they are also poisonous subcutaneously, when given by mouth they do not affect the health of the animals as is evidenced by the following. A rabbit weighing 3500 grams was kept without food for three days when through a soft tube introduced into its stomach one gram of finely powdered colon germ substance, suspended in water, was forced into the stomach by the use of a syringe. The animal was not visibly affected by this large dose either at the time or subsequently, though something like 50 mg. intraperitoneally would have killed it. More will be said later concerning the effects of the digestive ferments.

Experiment was made to determine whether or not the agent in the animal bodywhich breaks up the germ substances liberating the poison, is diffusible through collodion sacs and whether or not the poison thus set free is itself diffusible and can thus cause death. A collodion sac containing 1 gram of finely powdered typhoid germ substance suspended in 8 cc. of distilled water was inserted into the abdominal cavity of each of two medium sized rabbits about 3 o'clock in the afternoon. The rabbits showed no ill effect that evening from the operation.

At 7 o'clock the next morning No. 1 was found dead; No. 2 still showed no ill effect. The post-mortem of No. 1 showed the sac lying across the transverse colon and around it a very marked edema; the colon was hard, and there were various hemorrhagic spots in the omentum over it. Otherwise there were no abnormal conditions. No. 2 was found dead on the second morning. Autopsy showed a marked peritonitis as is also found after death from the intraperitoneal injection of the dead germ substance. In both cases the collodion sac remained intact after removal from the animal body. It may be inferred therefore that both the germ splitting agent and the separated poison are diffusible through collodion sacs.

Digestion with Pepsin and Trypsin.

The bacterial cellular proteins are, so far as their toxic properties are concerned, quite resistant to the action of pepsin and trypsin. It must be evident that in the study of this subject chemical work must be supplemented by physiological tests. A given sample of the cellular substance of the colon bacillus was tested upon a large number of guinea pigs in order to determine the minimum lethal dose. This was found to be for half grown animals 0.5 mg. given intraabdominally and 1 mg. subcutaneously. This material was then subjected for three days to an artificial gastric juice the efficiency of which was demonstrated simultaneously by its action on coagulated egg white. After three days the soluble and insoluble portions were separated and their toxicity tested. One half milligram of the undigested part given intraabdominally did not kill but 1 mg. did; while 1 mg. given subcutaneously no longer killed but 2 mg. did. Of the part that was dissolved in the acid pepsin solution, doses tested up to 100 mg. had no apparent effect. A like result was obtained from a similar study of the cellular substance of the typhoid bacillus. The amount of cellular substance left undigested after three days exposure to the acid pepsin was about 10 per cent of that originally taken. The conclusion that can be drawn is that the gastric juice slowly digests the bacterial cellular proteins and that in so doing destroys the poisonous group. With trypsin the effect is somewhat different. The cellular protein goes into

solution more rapidly and at least a part of the poison passes into solution without complete loss of its poisonous properties. The parts of both the colon and typhoid cellular proteins that passed into solution after three days' exposure to trypsin killed half grown guinea pigs in doses of from 35 to 40 mg. given intra-abdominally; while the undigested portion killed in doses of from 4 to 7.5 mg.

Cleavage with Dilute Mineral Acids.

Dilute acids slowly break up the cellular protein of the colon bacillus with at most only partial destruction of the poisonous group as is shown by the following:

One hundred milligrams of the cellular substance was boiled with 10 cc. of 1 per cent (by weight) hydrochloric acid. The protein was slightly reddened by this treatment and the mixture on standing separated into a reddish deposit and an opalescent supernatant fluid. The latter was filtered, carefully neutralized with sodium bicarbonate and injected into a guinea pig which died after some hours. In order to study the soluble products obtained by extraction with dilute acid at different temperatures the following experiment abbreviated from an earlier report (18) was made. Two hundred grams of typhoid cellular substance was extracted with 0.1 per cent sulphuric acid in the cold, then the residue at 100° and then at 120°. Four liters of the dilute acid was used at each operation and the extractions at a given temperature repeated so long as any part was removed, when the residue was again treated with acid, and extracted at the next higher temperature. This required three extractions in the cold, four at 100° and two at 120°. The extracts were precipitated with three volumes of 95 per cent alcohol, the precipitate filtered out, washed with alcohol, dried and powdered. The total amount of split product thus recovered amounted to approximately 20 grams or about 10 per cent of the 200 grams of cellular substance, while there were 26.96 grams of the residue finally remaining. The precipitates were purified by dissolving in water, so far as possible and reprecipitating with alcohol. In this way the split products were divided into a soluble and insoluble portion. The different soluble portions from each tempera-

ture were put together and repurified. The insoluble portions were saved except that at 100°. This was very small in amount and so slimy and mucilaginous that it was not possible to remove it from the filter. Determinations of ash, phosphorus and nitrogen were made in both soluble and insoluble portions, and likewise in the final residue and original germ powder. First the ash was determined, then the phosphorus in the ash. The nitrogen in each case was determined by the Kjeldahl method. The figures are given in Table 3.

TABLE 3.

Percentage Composition of Products from Typhoid Germ Substance with 0.1 per cent H_2SO_4.

	COLD.	100°.	120°.	RESIDUE.	GERM.
Soluble:					
Ash	72.6	5.28	3.02		
P	0.4058	0.385	0.87		
N	3.75	10.34	14.5		
N (ash free)	13.72	10.918	15.01		
Insoluble:					
Ash	55.65		4.78	2.60	5.7
P	8.43		1.50	0.21	1.85
N	5.89		12.26	13.04	11.55
N (ash free)	13.28	10.918	12.89	13.424	12.258

It is evident that the extraction in the cold brought down first of all a large part of the inorganic constituents of the germ, among which iron and sodium were found, and it is interesting that the split product which was the most toxic, the soluble part at 120°, was the one containing the largest percentage of nitrogen. These split products were more or less poisonous to guinea pigs but were evidently not the free poison as it took as long a time for them to kill as for the germ substance, that is, from 6 to 8 hours or even longer.

An ultimate analysis was made of the soluble part obtained at 100°, this being the only one of which there was a sufficient quantity for the purpose. The powder was dried to constant weight, and its constituents determined with the results given below. Carbon and hydrogen were determined by combustion, nitrogen by the Kjeldahl method; phosphorus in the ash by

precipitation, first with ammonium molybdate, then with magnesia mixture, sulphur by the alkali method (7) fusion with potassium hydrate and nitrate, solution of the residue in acidified water, and precipitation as barium sulphate, and oxygen by difference.

TABLE 4.

Typhoid Split Product at 100° with 0.1 per cent H_2SO_4.

	C.	H.	N.	S.	P.	O.
With ash (5.28 per cent)	45.73	5.95	10.34	1.13	0.386	36.46
Ash free.....	48.27	6.28	10.92	1.45	0.403	32.67

There was also an undetermined amount of iron.

The water solution of this split product gave good tests with the biuret, xanthoproteic and Millon protein reactions. There was also a Molisch reaction, indicating a carbohydrate group which was further supported by the fact that after boiling the substance with dilute acid, Fehling's solution was reduced. The split product was evidently protein in nature, though a compound protein.

It could not have been a protamin for the protamins are much richer in nitrogen, containing 30 per cent or more. A comparison of its percentage composition, calculated ash free, with the percentage composition of ash free animal protein as given by Hammarsten (8) is worthy of note, although a more extensive comparison with most of the known protein substances shows that its composition more closely resembles that of the group of glycoproteins, the mucin substances, especially that of a mucin from sputum, and a mucoid from tendon, as g ven by Cohnheim (9). The figures are given below. These glycoproteins, however, differ from the split product in that they contain no phosphorus. Only two phosphorized glycoproteins are known (8), namely ichthulin (10), occurring in carp eggs, and helicoprotein (11), obtained from the glands of the snail, and their percentage composition does not compare closely with that of the split product. Ichthulin has sometimes been classed with the nucleo-albumins. These phosphorized glycoproteins, like the split product, yield a reducing substance on boiling with acid.

TABLE 5.

Comparison Percentage Composition of Various Proteins with that of Typhoid Split Product.

	C.	H.	N.	S.	P.	O.	Fe.
Split product (ash free)	48.27	6.28	10.92	1.45	0.403	32.67	+
Animal protein (ash free)	50.6– 54.5	6.5– 7.3	15– 17.6	0.3– 2.2	0.42– 0.85	21.5– 23.5	
Mucin from sputum	48.26	6.91	10.7	+	–	33.1	
Mucoid from tendon	48.3	6.44	11.75	0.81	–	32.7	–
Helicoprotein	46.99	6.78	6.08	0.62	0.47		+
Ichthulin	53.52	7.71	15.64	0.41	0.43		0.1

On the whole the indications are that the split product obtained by the action of dilute mineral acid is of the nature of a phosphorized glycoprotein.

Blandine (12) reported the isolation of a nuclein and nucleoalbumin from culture of the typhoid bacillus, while Brieger and Fränkel (13), in their work on toxalbumins, found in cultures of the typhoid bacillus a protein which was toxic for rabbits and which was thought to contain the typhoid toxin in an impure state.

The Nitrogen of Three Acid Extracts. In order to study the distribution and amounts of the protein decomposition products as obtained through the action of acid, it seemed expedient to make a more general study of the nitrogen content of the germ as extracted by acid. To obtain some idea as to the amounts of amino, monoamino, and diamino nitrogen in the decomposition of the cellular substance by acid, represented by ammonia, monoamino acids and the hexon bases and to study their distribution when acids of different strengths were used, a comparative series was made, practically using the method of Hausmann (14). By this method he determined the amino nitrogen, determinable as ammonia, the nitrogen of the diamino bodies, as precipitated by phosphotungstic acid, and that of the monoamino acids as found in the filtrate not precipitated by phosphotungstic acid. The method though severely criticized by Kutscher (15) and others, has still been shown by Osborne and

Harris (16) to give valuable comparative results, whereby general differences are plainly evident. The method of procedure was as follows:

Three 10 gram samples of typhoid germ substance were extracted for five hours on the sand bath with reflux condensers, using 90 cc. of 1 per cent, 5 per cent, and 33⅓ per cent sulphuric acid, respectively, for the three samples. The residues were each then boiled for an hour with 100 cc. of water and this added to the extract, the whole being then diluted to 200 cc. The ammonia of each extract was determined by distilling with magnesia and the total nitrogen by the Kjeldahl method. The extracts were next each made to contain 5 per cent sulphuric acid, the required amount being added to the 1 per cent, and the 33⅓ per cent being diluted with the necessary amount of water. After this, each extract was precipitated with 25 per cent phosphotungstic acid, and allowed to stand 24 hours, when the precipitates were filtered out, washed with diluted acidified phosphotungstic acid, dried, weighed and powdered and the nitrogen determined by the Kjeldahl method. The nitrogen was also determined in the filtrates from these precipitates and in the dried residue remaining from the acid extraction. The results are given in Table 6. All the acid extracts gave the xanthoproteic and Millon reactions for protein, and all, save the 33⅓ per cent. the biuret test. The amount of ammonia nitrogen

TABLE 6.

Nitrogen as Ammonia-Typhoid.

	1 PER CENT.	5 PER CENT.	33⅓ PER CENT.
Amount of extract	200 cc.	200 cc.	200 cc.
Wt. N as NH_3 in 200 cc	0.0112 gms.	0.0112 gms.	0.0112 gms.
Total Nitrogen of Extracts (including N as NH_3).			
Amount of extract	200 cc.	200 cc.	200 cc.
Wt. N in extract	0.4284 gms.	0.7644 gms.	1.0164 gms.
Total Nitrogen Separated as NH_3.			
	2.6143	1.4652	1.1019

split off was the same in each case, which pointed to a definite cleavage regardless of the strength of the acid. The percentages varied, of course, since the total nitrogen of the extracts was different. The largest amount of total nitrogen was found in the 33⅓ per cent extract, and hence the smallest per cent of ammonia nitrogen, while the smallest total nitrogen was in the 1 per cent extract. The ammonia nitrogen varied from 1.1 per cent to 2.6 per cent. Hausmann (14) found approximately 1.2 per cent ammonia nitrogen in true proteins, which was in accordance with the results of other investigators.

TABLE 7.

Calculated for Typhoid Germ Substance (10 grams sample used).

	Per Cent
Total nitrogen	11.55
Ammonia nitrogen	0.112
Per cent of total N of germ as ammonia nitrogen	0.97

The total nitrogen of the germ was determined by the Kjeldahl method applied to samples of germ powder. The per cent of ammonia nitrogen was found from the fact that 0.0112 gram nitrogen was obtained from each of the three extracts from 10 gram samples.

TABLE 8.

Nitrogen of the Phosphotungstic Precipitates.

	1 PER CENT.	5 PER CENT.	33⅓ PER CENT.
Wt. ppt.	7.8220	12.8602	16.9792
Wt. N in ppt.	0.2014	0.3429	0.2029
Per cent N in ppt.	2.575	2.667	1.197

The largest amount of diamino nitrogen was found in the precipitate from the 5 per cent extract. It is probable that the 33⅓ per cent acid was strong enough to further decompose the diamino compounds and so lessen their quantity. In working for hexon bases a 5 per cent acid extract might be expected to produce the largest yield. The percentages of nitrogen in the precipitates from the 1 per cent and 5 per cent extracts were notably close together, and it is of interest that the 2.66 per cent nitrogen

found in the precipitate from the 5 per cent extract was quite close to the 2.68 per cent nitrogen calculated by Gulewitsch (17) in the phosphotungstic precipitate of arginin, but the carbon and hydrogen found were about twice what he calculated. His figures were C, 3.44 per cent; H, 0.74 per cent, while this precipitate contained C, 8.34 per cent, and H, 1.68 per cent.

TABLE 9.

Nitrogen of Filtrates from Phosphotungstic Precipitates.

	1 PER CENT.	5 PER CENT.	33⅓ PER CENT.
Total amt. of filtrate.....	200 cc.	260 cc.	260 cc.
Wt. N in whole filtrate.....	0.0315 gm.	0.07189 gm.	0.36218 gm.

The largest amount of monoamino nitrogen was found in the filtrate from the 33⅓ per cent extract; the smallest in that from the 1 per cent, showing that the weaker acids do not produce so complete cleavage as the stronger.

TABLE 10.

Nitrogen of Residue Left from the Acids.

	1 PER CENT.	5 PER CENT.	33⅓ PER CENT.
Wt. residue from 10 gms. germ substance..	3.9630	1.7322	0.7308
Wt. N in residue.....	0.4633	0.1924	0.0457
Per cent N in residue.....	11.6900	11.1064	6.2631

The per cent of nitrogen in the residues from the 1 per cent and 5 per cent acids was not greatly changed from that of the original typhoid germ substance (11.55 per cent), which would seem to indicate that the residue was still undecomposed.

Thus it will be seen that dilute acids slowly break up the cellular proteins; that about 10 per cent of the weight of the original germ substance may be recovered as split product; that the split product seems to be of the nature of a phosphorized glycoprotein; and that while the poison of the germ substance seems to be transferred in part, at least, in these split products, it can by no means be regarded as existing in anything but a combined

state as it still requires from 6 to 8 hours to kill an animal just as in the case of the cellular substance. Furthermore it appears that the acid cleavage of ammonia nitrogen from the germ powder of the typhoid bacillus is definite; that the largest amount of diamino nitrogen is obtained by the use of 5 per cent acid as compared with that obtained by the use of 1 per cent and 33⅓ per cent while the largest amount of monoamino nitrogen is found in the 33⅓ per cent acid extract.

Cleavage of Cellular Substance with Strong Mineral Acid.

Investigators thus far have looked very largely to a study of the decomposition products of the various albumins to gain an insight into their structural composition. The evident protein nature of the bacterial cellular substances leads to the inquiry as to whether or not the decomposition products of these germ complexes would correspond to those from better known proteins. Some light has been thrown upon the question by work done in this laboratory. Search has been made especially for two groups of bodies which have come to be regarded as essential constituents of all true albumins, namely the hexon bases and monoamino acids. Leach (1 and 2) has established the presence of lysin in the colon bacillus and shown the possibility of both histidin and arginin. Agnew (3) has obtained from the germ substance of both colon and tubercle bacilli monoamino acids as follows:

	Colon. Per Cent.	Tuberculosis. Per Cent.
Glutamic acid	3.00	0.2
Glycocoll	0.33	
Alanin	1.00	1.40
Valin	1.60	4.60
Leucin	2.00	1.82
Phenylalanin	0.20	0.50

Leach in one experiment extracted about 300 grams colon germ substance with 33⅓ per cent sulphuric acid and worked up the extract for hexon bases by the Kossel and Kutscher method (19) leaving a phosphotungstic filtrate to be investigated for monoamino acids. This was turned over to the writer and from

it both tyrosin and leucin were obtained as follows. From the solution, phosphotungstic acid was removed with barium hydrate and carbon dioxid used to remove excess of barium. By concentration and crystallization bodies were obtained resembling tyrosin and leucin under the microscope. These were purified by repeated recrystallization from water or from ammoniacal water, the tyrosin being so much less soluble than the leucin that they could be separated by difference of solubility. It was necessary to boil the leucin fraction with animal charcoal to remove coloring matter. The tyrosin formed the characteristic colorless silky needles, many grouped in the characteristic sheaves. As it became more pure the needles were longer and larger and grouped in the sheaves less persistently. After many purifications the crystals melted at a constant temperature, though this was difficult to determine since tyrosin melts with decomposition. The melting point maintained after each of two or three recrystallizations was 288° uncorrected. The correction was 8.13° which made the corrected point 296.13°. A Kahlbaum preparation of tyrosin in the laboratory melted within a degree of the same point and agreed in the chemical tests to be mentioned presently. Richter gives the melting point of tyrosin as 235°, Cohn as 295°, while Fischer says that with rapid heating the corrected point is 314° to 318°. The tyrosin obtained gives a Hofmann's test with Millon's reagent. It gives Scherer's test with nitric acid and sodium hydrate on platinum foil and also a beautiful Piria test with sulphuric acid, then barium carbonate and ferric chloride, which test is characteristic for tyrosin.

The leucin crystallized in the characteristic knobs or balls. As it became purer it crystalized more and more in shining white very thin plates, sometimes in radial groups, sometimes not. The crystals were finally obtained with a practically constant melting point 262° to 263° or corrected 268.6° to 269.6°. The pure laboratory leucin (Kahlbaum) melted at the same point. Schwanert, Hammarsten and others give the melting point for active leucin as 170°. That for the inactive form is given as 270°. Fischer says the melting point is 293° to 295° (corrected) if heated quickly in a closed tube. Cohn gives 275° to 276°. The leucin obtained melted with darkening and decomposition. With careful heating in an open tube it sublimed with the characteris-

tic white, wooly deposit. It also responded to Scherer's test on platinum foil with nitric acid and sodium hydrate which test Hammarsten says is characteristic for leucin.

Cleavage of the bacterial cellular substances with strong mineral acids, yields then, both monoamino and diamino acids, thus giving another proof of true protein composition.

Cleavage with Alkali in Water Solution.

With dilute alkali in the cold, 0.5 per cent potassium hydrate with the colon bacillus and both 0.5 and 5 per cent potassium hydrate with sarcina lutea, very little is removed from the cell as has already been shown by the writer (20). Heat is necessary to effectively split the molecular structure. When heated with dilute alkali the extracts obtained are thick, syrupy liquids containing a deposit of a slimy, mucilaginous nature which makes filtration exceedingly difficult, the method proving most satisfactory being the use of several thicknesses of hardened paper without the pump. These water alkaline extracts give all the protein color reactions, including a strong Molisch carbohydrate test and the reduction of Fehling's solution after boiling with dilute mineral acid. Excess of alkali or inorganic salt often interferes with the Millon test which is obtained perfectly upon removal of the alkali or upon sufficient dilution.

In one instance the colon germ substance from six incubating tanks, about 300 grams, was heated on a boiling water-bath with 6 liters of a 2 per cent potassium hydrate solution, the extract filtered through folded papers, and acidified with acetic acid. The precipitate produced, presumably protein, and filtered out after standing twenty-four hours was so small in amount that it was lost in the filter paper. Three volumes of 95 per cent alcohol for every volume of acidified extract were then acidified with hydrochloric acid to the amount of 0.5 per cent and into this acid alcohol the acidified extract was poured, producing a heavy white, curdy, fibrous precipitate, which was filtered, washed acid free with alcohol and then washed with ether. After purification by solution in one liter of 0.5 per cent potassium hydrate, reprecipitation in three liters 95 per cent alcohol, washing as before with alcohol and ether,

there remained 25 grams of the white, flaky precipitate, something less than 10 per cent of the original germ powder. Injections of solutions of this precipitate up to 125 mg. had no effect upon guinea pigs showing that the poison was not there. The possibility of having obtained a nuclein or nucleic acid naturally suggested itself. Determination of ash and phosphorus showed 5.9 per cent of ash and 0.194 per cent of phosphorus. Among the protein color reactions this split body gave the xanthoproteic alone and that none too well. With α-napthol and sulphuric acid it gave a beautiful furfurol ring (Molisch carbohydrate test) and when boi ed with dilute mineral acid it reduced Fehling's solution with great readiness. The low percentage of phosphorus showed that it could not be a nuclein or nucleic acid while the other tests pointed to the presence of a carbohydrate. To try the possibility of further splitting the body a 1 per cent (by weight) sulphuric acid extract was made in the cold, the extract filtered and precipitated with 95 per cent alcohol It was thought that there might thus be obta ned a precipitate with a higher phosphorus content. Instead, the three parts of the first precipitate, that is, the precipitate and filtrate of the acid extract and the part remaining from the acid extraction, gave only carbohydrate tests. As the primary search was for the poison this carbohydrate was not identified at this time.

From the first alcoholic filtrate from this carbohydrate, in other words, from the alcohol soluble part of the 2 per cent potassium hydrate extract of the colon germ substance, there was obtained, after evaporaton of all liquid, resolution in absolute alcohol to remove inorganic salts and evaporation of the alcohol, a black, gummy, sticky, viscous mass which defied every attempt at purification and every effort to put it into a solid, weighable form, but which gave perfectly the protein color reactions. In this black, sticky mass, containing apparently a protein soluble in absolute alcohol, was found also a poison, small unweighed doses killing animals in 6 or 8 minutes, which was quite different from the 6 or 8 hours necessary to a fatal issue with the cellular substance and which indicated the poison in a more free or less combined state so that its physiological effect was obtained at once without the time interval necessary for the body juices to act upon the complex structure of the

cellular molecule and thus to liberate the poison. Various efforts to extract the poison from its viscous host with ether, chloroform, petroleum ether, amyl alcohol, etc., were tried to no avail. Ammonium sulphate, to saturation, salted out only a very little protein and produced no separation. Mercuric chloride and lead acetate gave excellent precipitates but in each case, after removal of mercury and lead from their respective precipitates and filtrates, the poison remained always in the filtrates. Phosphotungstic and phosphomolybdic acids produced precipitates but no other indication of alkaloidal bodies. It seemed necessary for further progress to obtain the poisonous portion in a state of greater purity and if possible free from the sticky carbohydrate. Hydrolysis with dilute sodium hydrate in both water and absolute alcohol solution was tried, sodium being used to replace potassium in order to avoid the possible presence of poisonous inorganic salts. By extracting with 1 per cent alkali, no poison was obtained in either water or alcohol, but when the sodium hydrate was increased to 2 per cent as before, both water and alcoholic solutions contained a highly poisonous body. The decided advantage however lay with the alkali-alcohol extraction, in that by this method a larger proportion of toxic material was obtained; it was possible to get rid of most of the inorganic constituents by simply acidifying with hydrochloric acid and filtering out the sodium chloride precipitated in the alcohol and lastly all of the carbohydrate remained in the insoluble residue so that by careful distillation and evaporation, both in vacuo, of the alcohol from the neutralized extract, the poisonous substance was obtained in a hard brittle mass which could be ground to a fine powder. The poisonous portion of the cellular substance as extracted by dilute alkali in absolute alcohol will be discussed in detail after mention is made of the effect of strong alkali in water.

Strong alkali was used to find out whether or not it would separate the poison in a still simpler or less combined form. Colon germ substance was boiled on the sand-bath for four hours with strong sodium hydrate (1 : 2). During the process large quantities of ammonia were given off. At the end of this time the black, rather thick solution was diluted with an equal volume of water and filtered. By far the larger part

of the cellular protein had gone into solution, only a small residue remaining. The clear filtrate was acidified with hydrochloric acid, giving off a strong indol odor though no test for indol could be obtained. The solution was next evaporated to dryness. The dry substance gave no biuret test, but still showed the Millon and Molisch reactions. It burned with practically no ash and contained both nitrogen and sulphur, the presence of phosphorus being doubtful. A portion of this dry substance, extracted with absolute alcohol, filtered, evaporated, redissolved in water and neutralized with sodium bicarbonate was somwhat poisonous to guinea pigs but not highly so, indicating that the poisonous action was diminished rather than increased by the use of strong alkali.

Cleavage with Dilute Alkali in Alcohol.

The first work upon the cleavage with dilute alkali in alcohol was done with the bacterial cellular proteins but later the method was applied to other proteins of both animal and vegetable origin. The process, which is the same for any protein, has already been described in detail in a previous paper (21), having been in use in this laboratory for several years, but may be outlined for the sake of clearness. The dry cellular substance or the protein from whatever source, prepared as has been described in the form of a fine powder, is boiled for an hour on the water-bath, under a reflux condenser, with from fifteen to twenty-five times its weight of absolute alcohol, in which 2 per cent by weight of pure sodium hydrate has been previously dissolved. It is essential that the alcohol used in this extraction should be absolute, and that every trace of water in the substance should be removed before the extraction of the poison is undertaken. If this point be neglected the poison will be obtained in the same sticky, gummy mass as with the water-alkali extraction and with which satisfactory results are impossible. By this process the germ substance or the animal or vegetable protein is divided into two distinct parts. From the golden-brown alcoholic solution there is obtained a powder representing about one-third of the original dry germ substance and a somewhat larger proportion of the other proteins, which is

very toxic, killing guinea pigs in small doses in about thirty minutes, while the residue insoluble in alcohol, represents about two-thirds or less of the weight of the original powder according as to whether obtained from cellular substance or from a more simple protein, and is not poisonous. The protein does not yield all its poison, as a rule, until the extraction with sodium hydrate in alcohol has been repeated at least three times. Boiling the bacterial, animal or vegetable protein thus with sodium hydrate in alcohol splits the molecule along certain definite lines of cleavage, for when the extraction has been repeated several times, boiling for an hour at each extraction, it will be found that all the poison is dissolved in the alcohol, that no more can be split off, and that the portion insoluble in alcohol has no longer toxic properties. After these portions have been separated by filtration, the filtrate, containing the poison dissolved in alkaline absolute alcohol, is neutralized with a calculated amount of hydrochloric acid. This throws down practically all the base and acid as sodium chlorid, which is removed by filtration. There is thus obtained a solution of the poisonous part of the germ cell or other protein in absolute alcohol. The greater part of the alcohol is distilled in vacuo under 40° and the evaporation continued to dryness in vacuo at 40°. Resolution in absolute alcohol removes remaining traces of sodium chlorid, the solution being again evaporated to dryness in vacuo at 40°. The poisonous part of the protein obtained in this way provided there is no water present, forms a brown scale on the dish, which may be pulverized to a brownish powder that is toxic in small doses.

It is believed that this poisonous substance is an integral part of the bacterial cell or other protein, hydrolyzed from it by the alkali, and that it is a synthetic product formed during the protein's growth. If the poison is not obtained from the germ cell, the only other source possible is from some part of the culture medium. In this case it would have to come either from the beef tea or from the agar. To test the possibility, 200 cc. of beef tea were poured into five volumes absolute alcohol. After settling, the precipitate was filtered out, dried and weighed. There were 1.5 grams. Ordinarily, when beef tea cultures are employed to inoculate the tanks from 500 to 600 cc. are used.

This would mean that if all the beef tea remained at the harvest and were removed with the bacterial growth that there would be a possibility of precipitating by the alcohol along with from 200 to 300 grams of germ substance, at most from 3.75 to 4.5 grams of foreign substance. As a matter of fact it must be very much less, the beef tea being exhausted by the growth of the germ, so that it will readily be seen that this source could not yield some 70 to 100 grams of poison. As to any possibility of agar as a source of the poison, 40 grams of agar were cut very fine, extracted on the water bath with 2 per cent sodium hydrate in absolute alcohol, dried, powdered in a mortar, and reëxtracted with sodium hydrate in absolute alcohol. The yellow alkaline extracts, filtered from the insoluble residue, were just acidified with hydrochloric acid, the salt filtered out, and the clear solution evaporated to dryness. The dry residue was very small in amount, mostly insoluble in water, showed none of the protein color reactions, and had no effect whatever upon animals, thus eliminating agar as a source of error. On the positive side of the question to show that the poison is an integral part of the cell and built up synthetically during its growth from the elements of the cultural medium, the colon germ was grown on Fraenkel's modification of Uschinsky's medium, which contains no protein whatever, as such. After a week's development the contents of the flask were poured into from two to three volumes of 95 per cent alcohol, the precipitated substance filtered out and put into absolute alcohol. Filtered from this it was extracted in Soxhlets with ether, dried and powdered. From this germ substance the process for the extraction of a poisonous body with 2 per cent sodium hydrate in absolute alcohol was carried out as has been outlined. On concentration of the alcoholic solution there was obtained a poisonous body just as from the extraction of germ substance from agar grown cultures. The same dose killed guinea pigs in thirty minutes, with characteristic symptoms of colon poison. Also, the poison obtained from germ grown on Fraenkel's modification of Uschinsky's medium gave all the protein color tests described later as being obtained from colon poison from cultures grown on agar. This demonstrates that the poison is an integral part of the germ split off from the cell by alkali, and in consideration of its apparent

protein nature, set forth in detail subsequently, it is evident that the bacterial cell must synthetically produce this protein body during its growth from the chemical components of the medium.

Considering the animal and vegetable proteins, aside from those of bacterial origin, it is evident that here the bodies are obtained in a purer and more simple form and that what is split off by the alkali must be produced from the substance itself. Also it seems logical to conclude that the line of cleavage obtained by the use of alcoholic alkali must somewhat nearly approximate the physiological process, since after sensitization, an injection of the whole protein kills with precisely the same symptoms as are obtained with a fatal dose of the split off poison.

Ammonia Set Free by Cleavage with Dilute Alkali. During the extraction of the protein poison with dilute alkali the odor of ammonia is apparent even at the end of the third extraction. An effort was made to discover how much nitrogen was converted into ammonia. A device was arranged for conducting the ammonia into standard acid, $\frac{N}{10}$ sulphuric being used, and four ten gram samples of Witte peptone were extracted with 2 per cent sodium hydrate in absolute alcohol, one for 3 hours in a current of air the other three in a current of hydrogen for 2.5, 8.5 and 19.5 hours respectively. At the end of each operation the excess of acid was titrated with $\frac{N}{10}$ sodium hydrate and the per cent of nitrogen calculated. The toxicity of the split product was also determined. In every case ammonia was still being produced when the process was interrupted. Again, a ten gram sample of albumin poison was boiled for 54.5 hours with the 2 per cent alcoholic alkali to find out whether ammonia could be split from the poison itself and the effect. The table below shows the results.

TABLE 11.

Ammonia Produced by Cleavage of Protein with Dilute Alkali in Absolute Alcohol.

SAMPLE.	TIME IN HOURS.	ATMOSPHERE.	PER CENT N OF SAMPLE GIVEN OFF.	RATE N PER HOUR IN PER CENT.	TOXICITY OF POISON.
Witte Peptone.....	3	air	0.4305	0.1435	Diminished.
Witte Peptone.....	2.5	H	0.3956	0.1582	Increased above that extracted in air.
Witte Peptone.....	8.5	H	0.7383	0.0868	
Witte Peptone.....	19.5	H	1.0517	0.0539	Between that of the first two above.
Albumen Toxin.....	54.5	H	1.48	0.027	Diminished by half.

Albumin toxin as ordinarily extracted contains 13.74 per cent of nitrogen. By the 54.5 hours heating with alcoholic alkali 10.77 per cent of its nitrogen was converted into ammonia. After this treatment the poison still gave a good Millon test but no longer a biuret. It is probable that by continued heating in the same manner quite all of the nitrogen could be separated though it is noticeable that the rate per hour was gradually diminished as the time lengthened.

Properties of the Non-Poisonous Portion.

It is not within the province of this paper to speak at length of the non-poisonous portion of the protein molecule, a number of papers having already appeared upon the subject from this laboratory, from its chemical standpoint by Leach (22) and Agnew (3), from its physiological side by Vaughan (23), V. C. Vaughan, Jr. (24), J. W. Vaughan (25) and by Vaughan and Wheeler (26) jointly. Suffice it to say that the non-poisonous portion contains the specific part of the protein, the part that immunizes and sensitizes. It gives the protein color reactions, the Millon test rather poorly; it contains most of the phosphorus and all of the carbohydrate. Percentages of nitrogen and ash

in a number of these residues, determined by Gidley and Houser in this laboratory, not published elsewhere, save two by Leach, may be of interest.

TABLE 12.

Nitrogen and Ash in Non-poisonous Portion of Proteins.

RESIDUE.	PER CENT N.	PER CENT ASH.	PER CENT N ASH FREE.
Colon (Leach)	5.56	26.08	7.52
Tuberculosis	7.295	23.36	9.53
Typhoid	5.955	16.50	7.13
Anthrax	2.43	46.73	4.56
Pyocyaneus	7.29	27.25	10.02
Proteus Vulgaris	5.99	25.29	8.02
Ruber of Kiel	5.58	15.49	6.60
Subtilis	7.00	21.63	8.93
Megaterium	4.855	18.50	5.95
Albumen (Leach)	12.67	13.57	14.53
Casein	8.73	19.80	10.88
Edestin	13.125	15.19	15.48
Serum Albumen	6.995	32.27	10.26
Witte Peptone	10.38	19.90	12.98
Macquaire Peptone	7.185	32.73	10.63
De Chapoteaut Peptone	6.78	41.46	11.58

A large part of the ash is composed of inorganic salts due to presence of the sodium hydrate which can be completely removed from the residue only by dialysis.

General Properties of the Poisonous Portion.

The poison split from the protein molecule by hydrolysis with dilute sodium hydrate in absolute alcohol is undoubtedly crude. It is probable, as the ammonia determinations show, that the process by which it is obtained serves also to destroy or diminish it if carried too far. But crude as it is, there is present a poisonous body, fatal to guinea pigs intraperitoneally in doses of from 8 to 60 mg. according to its purity and in from 30 to 60 minutes, or even in shorter time if the dose be large, and the symptoms produced are identical in kind, in sequence and relative duration whatever the protein from which the poison is obtained. The

physiological action of the poison has been presented by V. C. Vaughan, Jr. (27), and will not be further discussed here except to say that by mouth it is not poisonous, a fasting rabbit bearing a whole gram of typhoid toxin introduced into its stomach by means of a tube, without apparent effect.

So far a large number of protein bodies bacterial, animal, vegetable, have been split up and no true protein as yet has failed to yield a poisonous group. Among the poisons obtained are those from Bacillus coli communis, Bacillus typhosus, Bacillus anthracis, Bacillus tuberculosis, Bacillus mœlleri (timothy), Sarcina lutea, Bacillus ruber of Kiel, Bacillus proteus vulgaris, Bacillus subtilis, Bacillus megaterium, Bacillus pyocyaneus, from egg albumen, casein, serum albumen, edestin, zein, Witte peptone, Macquaire peptone, De Chapoteaut peptone and from the tissue of two cancers. Gelatin contains no poison, but gelatin is an albuminoid, and scarcely gives the Millon test, if at all. Witte's peptone contains a poison as does also that of Macquaire and De Chapoteaut, but Nicolle and Abt (28) have found that Defresne's peptone does not and this we have confirmed. However, we do not know whether this is made from gelatin or from a true protein.

The brownish, poisonous powder is very hydroscopic and dissolves freely in water, forming an opalescent solution, acid in reaction, and pale yellow to golden brown in color according to its strength. The opalescence is removed by filtration through a hardened paper, but the toxicity of the solution is not thereby diminished, showing that a small but non-poisonous part of the powder is insoluble in water, but the larger part is so freely soluble that for practical purposes the opalescence may be disregarded. On neutralizing the opalescent solution with sodium hydrate it becomes clear; on neutralizing with sodium bicarbonate, a brownish, non-toxic precipitate is formed, small in amount, and when this is removed by filtration the solution obtained is clear and highly toxic. In absolute alcohol the solution is perfectly clear. After long standing in an absolute alcohol solution, there is a small part that settles out and becomes insoluble; also, after repeated evaporation to dryness and resolution in absolute alcohol, a small part each time becomes insoluble. The powder is soluble in methyl as well as ethyl alcohol but not in amyl

alcohol, and is insoluble in ether, chloroform and petroleum ether. Each of these latter will remove a very small amount of fatty substance which is non-toxic, but they do not dissolve an appreciable amount of the poison. The powder is soluble in strong mineral acids remaining clear on being boiled and on diluting with water. A few drops of mineral acid, however, added to a water solution, causes a precipitate, which would seem to indicate that the acidity of the water solution of the poison is caused by the presence of some organic acid.

The poison diffuses slowly through collodion sacs both within the animal body and suspended in distilled water, as is shown by the following. Two hundred milligrams of typhoid poison dissolved in 20 cc. of water was placed in each of two collodion sacs which were then suspended in distilled water. At the end of 24 hours the Millon reaction was given by the dialysate. This was replaced every 24 hours by distilled water and the dialysis continued for 96 hours. At the end of this time the combined dialysates were concentrated to dryness, dissolved in absolute alcohol and again evaporated to dryness. The brown residue, sticky in appearance, dissolved in water was acid in reaction, had the characteristic "toxin odor," and killed a guinea pig in 20 minutes showing that the poison is diffusible. So slowly, however, does it diffuse that even at the end of the 96 hours it was not yet entirely removed from within the sac. In another experiment a collodion sac containing one gram of typhoid toxin suspended in 8 cc. of water was put into the abdominal cavity of a medium sized rabbit. There resulted no visible effects upon the animal. After 12 days the sac was removed. In the sac, which was intact, were found 6 cubic centimeters of a clear fluid, which looked more like blood serum than anything else. Three cubic centimeters of this fluid had not the slightest effect upon a guinea pig showing that the toxin had undoubtedly diffused but had evidently diffused so slowly that there was not enough present at any one time to affect the health of the rabbit.

Although the toxic powder is so readily soluble in absolute alcohol, the tests point to the presence of an albuminous or protein body. Practically all the protein color reactions are obtained, with the exception of Molisch's test. It is interesting,

too, that the part separating out from an alcoholic solution on long standing gives none of the protein color reaction, indicating that the protein part is permanently soluble in absolute alcohol. The Millon's reaction shows most perfectly and persistently wherever the poison is found. Mann (29) says that this reaction is given by all benzene derivatives in which one hydrogen atom has been replaced by the hydroxyl group OH and as, according to Salkowski (30) and Nasse (31), tyrosin is the only oxyphenyl compound in protein, the reaction is presumed to show the presence of tyrosin. The biuret reaction, not obtainable with any but the albuminous dissociation-products of protein and used to distinguish between albumins and their simpler or secondary decomposition products, is given most beautifully. Heated with strong nitric acid a clear pale yellow solution is formed, which when ammonia to excess is added, turns to a deep orange color, thus giving the xanthoproteic test and indicating the presence of aromatic radicals. The reaction of Adamkiewicz is positive, for when the poison is heated with acetic acid or, as Hopkins and Cole (32) suggest, shaken with glyoxylic acid, and then strong sulphuric acid is added, a beautiful violet color develops. Hopkins and Cole (33) have shown that this reaction depends on tryptophan or indol-amino-propionic acid. On boiling with concentrated hydrochloric acid, to which a drop of concentrated sulphuric acid has been added, the powder passes into solution and a violet color results, thus giving Lieberman's test, which was considered by Hofmeister (34) to be a furfurol reaction in which furfurol produced from the carbohydrate radical of the albumen molecule and the aromatic oxyphenyl radical took part, though Cole (35) asserts that it is due "to an interaction between the glyoxylic acid which is present in the ether used for washing the albumin, and the trytophane which is split off from the albumin by the action of concentrated hydrochloric acid." However this may be the poison does not respond to the Molisch test, given by the formation of furfurol on the splitting up of a carbohydrate group with sulphuric acid, nor does it reduce Fehling's solution either directly or after boiling with dilute mineral acid.

The ordinary test for sulphur in proteins, that of heating with excess of sodium hydrate in the presence of a small amount of

acetate of lead, s not given by the portion of the protein split off by alkali in absolute alcohol. If, however, a portion of the substance in a test tube is fused with metallic sodium and the cooled mass treated with water, a few drops of a freshly prepared solution of sodium nitroprussiate added to part of the clear filtrate give a beautiful violet color, indicating the presence of sulphur. Also, if the other part of the clear filtrate be treated with a lead acetate solution and acidified with acetic acid, lead sulphid is precipitated. If the solution is acidified before lead acetate is added a faint but unmistakable odor of hydrogen sulphid is detected. It is known that sulphur may exist in the albuminous molecule in at least two forms, one part being readily split off on heating with dilute alkalies as hydrogen sulphid, the other being obtained only when the destruction of the protein molecule is carried much farther. Since the nitroprussiate reaction is very delicate, no conclusion as to the amount of sulphur can be drawn from this test, and although a good precipitate of lead sulphid is formed the amount of sulphur is probably not large, since Leach failed entirely to find sulphur present in the ash of the colon bacillus, though both the germ substance and the residue which remains after extraction of the poison, respond to the nitroprussiate test for sulphur, and also give the lead sulphid precipitate in the clear acidified filtrate from the fused mass.

A solution of the toxic substance is not coagulated by heat in acid, neutral or alkaline solution, though, as already stated, a few drops of a mineral acid causes the appearance of a considerable precipitate which is not soluble on heating or on the further addition of acid. This precipitate is produced regardless of whether or not the opalescence of the water solution is first removed.

Among the metallic salts, copper sulphate produces no precipitate and ferric chloride only on heating. Silver nitrate naturally precipitates any trace of chlorid present, but after the addition of an excess of ammonia there still remains a small precipitate. Potassium ferrocyanid gives a precipitate, also potassium bismuth iodid in acid solution. Lead acetate, mercuric chlorid and platinum chlorid all produce heavy precipitates. With lead acetate and mercuric chlorid, however, after removal

of lead and mercury with hydrogen sulphid from their respective precipitates and filtrates, the protein reactions are given by the filtrates and here also is found the poison in each case. From 10 to 15 per cent of the crude poison can be precipitated by the use of platinum chlorid in either water or alcoholic solution. All attempts to crystallize this precipitate failed as only a small part of it is dissolved by hot water and the insoluble part is unaffected by any of the other ordinary solvents. The protein reactions are given by the platinum precipitate by both soluble and insoluble parts, but not by the filtrate. The poison is found in the insoluble part of the precipitate after removal of the platinum by hydrogen sulphid, its toxicity being markedly increased. The other parts, after removal of the platinum are inert.

From a water solution of the poison, bodies giving protein reactions may be salted out by the addition of amonium sulphate or sodium chlorid to saturation, but in neither case is the separation complete, the filtrate still responding to the protein color tests after removal of the neutral salt. In the case of salting out with ammonium sulphate, the solubility of both parts is thereby lessened and the toxicity diminished, possibly on account of decreased solubility, though both parts exhibit some poisonous action and likewise both show the protein color tests.

Phosphotungstic, phosphomolybdic and picric acids all give abundant precipitates. Since these reagents are also used in the precipitation of alkaloidal bodies, the precipitates with phosphomolybdic and phosphotungstic acids were further examined, the possibility suggesting itself that the toxic body might be alkaloidal in nature and that the protein part might be entirely separate from the poison. A sample was precipitated with phosphomolybdic acid in acid solution, the precipitate removed, washed and dissolved in ammoniacal water. This solution was then shaken with amyl alcohol but the alcohol was not colored and the residue obtained on concentration was so slight as to be practically nothing. Another sample was precipitated with phosphotungstic acid, the solution being acid in reaction. The precipitate was allowed to settle, removed by filtration, washed with acidulated water, decomposed with a saturated solution of barium hydrate and the remaining insoluble part filtered out. So far

as possible the barium was removed from the filtrate with carbon dioxid, alternating with concentration and further addition of carbon dioxid. The solution was then allowed to concentrate to dryness, when the residue was dissolved in absolute alcohol, leaving barium salts behind.

On concentrating the slightly opalescent solution, more barium salts came down during the process and were filtered out. The dry residue was taken up in water and ammonium carbonate used to precipitate the barium that still remained. After removing the barium carbonate, by evaporating on the water bath, both carbon dioxid and ammonia were expelled, the solution again becoming acid. Dryness being reached absolute alcohol was once more used, leaving undissolved a small amount of inorganic material. In this way the final residue after evaporation of the alcohol was practically freed from inorganic impurities. Sulphuric acid no longer gave a barium precipitate in water solution. The amount obtained by this method was very small and an exceedingly small part of the original toxic powder. Since the substance obtained in this way still gave good Millon, biuret and xanthoproteic protein reactions, it is fair to say that it was not alkaloidal. The very small amount obtained by this method given to a guinea pig intra-abdominally made the animal sick but did not kill. Either phosphotungstic acid does not precipitate the toxic body or else the amount obtained was less than a fatal dose.

Should the poison contain an alkaloidal body existing as a salt in the acid solution, the possibility of extracting the base with ether or chloroform after the solution had been made alkaline with ammonia, is apparent. This was tried with negative results. To a water solution of colon poison, acid in reaction, ammonia was added, drop by drop, to a slightly alkaline reaction, the mixture shaken with ether, the ether separated and evaporated. The residue remaining was non-toxic. The ammoniacal water solution was next shaken with chloroform, the slightly colored chloroform drawn off and evaporated at low temperature leaving a small amount of a dark, thick semi-liquid, which was not poisonous either as it was or after faintly acidifying with hydrochloric acid. The water solution remaining being still poisonous, it is evident that the toxic part is not an alkaloidal body capable of being extracted directly.

Potassium bismuth iodid in acid solution of the crude soluble poison produces an abundant precipitate, apparenty more or less soluble in excess, and soluble in ammoniacal water.

Kowalewsky (36) has shown that uranyl acetate will completely remove from various albuminous fluids every trace of protein giving a biuret reaction while Jacoby (37) and others have used this reagent for the removal of proteins from faintly alkaline solutions. Abel and Ford (38) used it to remove protein from an extract of poisonous fungi. In a slightly alkaline solution of albumen poison, uranium acetate gave an abundant precipitate but not a complete separation as both precipitate and filtrate still gave the Millon and biuret tests and the filtrate, after removal of excess of uranium with a solution of disodium hydrogen phosphate, filtration, evaporation, solution in alcohol and reëvaporation, was still poisonous. In acid solution, the precipitation was complete, the filtrate no longer giving the protein reactions.

Freshly prepared metaphosphoric acid also produced an abundant precipitate but not a complete separation, the filtrate showing both Millon and biuret reactions. Likewise a heavy precipitate is produced by the use of a saturated solution of picric acid but the poison is not in the precipitate which gives only a very poor Millon test after removal of the picric acid and no biuret.

Hofmeister (39) has given a method for introducing iodine into the molecule of egg albumen. This was tried with the poison split from egg albumen. The iodized compound no longer gave either the Millon or biuret reaction, and while it affected animals more or less, they did not die and the symptoms were not those induced by toxin poisoning. The iodine seemed to have entered into chemical combination in the toxin molecule and to have thus changed its characteristics. The iodized body was freely soluble in absolute alcohol and in alkaline water, not in water alone and was precipitated by acid water from alcoholic solution, also on acidifying an alkaline solution. Though it no longer responded to the Millon and biuret reactions, a good test for nitrogen was obtained after fusing with metallic sodium.

An attempt was made to benzoylate the poison by the Schotten-Baumann method using albumin toxin. Practically no

precipitate was obtained. From the filtrate in a part soluble in hot alcohol there were obtained shiny, glistening plates or flat needles which matted together under suction and had much the appearance of some of the fatty acids. These were insoluble in water or very difficultly so if at all, difficultly soluble in cold alcohol, readily in hot. They gave no Millon test, no biuret, no Molisch and contained no nitrogen. After recrystallization from alcohol they melted constantly at 62°. Palmitic acid melts at 62° (Mulliken) and boils at 339° to 350° (Mulliken) A Merck preparation of palmitic acid melted at 62° and boiled at about 345° to 350°. The toxin crystals had not yet boiled at 360° though above 300° there was some decomposition. From the remainder of the filtrate there was obtained from the part soluble in cold alcohol a non-crystallizable body giving both Millon and biuret tests and containing 9.335 per cent of nitrogen, and from the part soluble only in water, likewise a non-crystalline compound, with 9.66 per cent of nitrogen and showing both Millon and biuret tests but not seriously affecting animals in a usual dose.

The nitrogen in a number of the toxins has been determined by Gidley in this laboratory as follows:

TABLE 13.

Percentage of Nitrogen in Protein Poisons.

Source of Poison.	Percentage of Nitrogen in Poison.
Colon bacillus	13.49
Typhoid bacillus	11.52
Tubercle bacillus	11.00
Pyocyaneus	10.50
Ruber of Kiel	10.495
Subtilis	8.12
Megaterium	8.595
Proteus Vulgaris	10.17
Yellow Sarcine	6.145
Egg Albumen (Leach)	13.74
Serum Albumen	10.48
Edestin	12.78
Zein	10.69
Witte Peptone	11.14
De Chapoteaut Peptone	12.735

To study the distribution of the nitrogen, determinations were made in both the colon and albumen toxins, of the ammonia nitrogen, the monoamino and diamino nitrogen by the method already described under cleavage with dilute mineral acids.

The following are the results.

TABLE 14.

Distribution of Nitrogen in Protein Poisons.

SOURCE OF POISON.	TOTAL PER CENT N OF POISON.	TOTAL PER CENT N OF ACID EXTRACT.	PER CENT AMMONIA N.	PER CENT MONAMINO N.	PER CENT DIAMINO N.
Colon bacillus	13.49	10.185	1.525	6.472	1.753
Egg albumen	13.74	11.477	0 745	7.999	1.40

It will be seen that the greater part of the nitrogen is to be found in monoamino combination. From the phosphotungstic filtrates from both the albumen and colon poisons containing the monoamino acids, crystalline bodies were obtained. Judged by the strong Millon test, tyrosin was undoubtedly present, but the crystalline masses were largely leucin, and no tyrosin was obtained in purified form. From the crude crystals, after many and repeated crystallizations what was thought to be leucin was obtained pure, melting at 264°–265° uncorrected or 269.42°–270.46° corrected. The crystals were thin plates characteristically grouped and sublimed readily. This will be referred to again later. From another 5 per cent sulphuric acid extract of albumin toxin was obtained a large mass of crystals in characteristic tyrosin-like sheaves and giving a deep Millon reaction. These were undoubtedly tyrosin though at the time no melting point was taken.

Cleavage of the Poisonous Portion with Strong Mineral Acids.

Up to this point every attempt to separate the poisonous body as a chemical unity had been unsuccessful, though each time some new point was added to its chemistry. A still further effort was made. The physiological effect produced by the crude

soluble protein poisons so nearly resembles that of neurin that repeated trials were made to isolate neurin or a neurin-like body. Typhoid toxin, colon toxin, and that from egg albumen and Witte's peptone were all investigated. They were all hydrolyzed with concentrated hydrochloric acid according to Brieger's method (40) and attempts made to form both the mercury and platinum salts. Crystals of various kinds, in exceedingly small amounts, were obtained but none of them poisonous, so that after many repetitions of the process, only one conclusion was possible, namely, that neither neurin nor similar bases were present in these poisons. Before this conclusion was reached, however, owing to the neurin-like physiological effect of the poison in causing death by paralysis of the respiratory centers and in view of the well known antagonistic action of atropin to neurin, the effect of atropin with the poison was tried. Guinea pigs were treated with small doses of atropin sulphate and later given a fatal dose of the Witte peptone toxin, others likewise the colon toxin with control animals treated respectively with poison and atropin. In no case did the atropin itself kill; in all cases the control toxin pigs died. With the peptone toxin previously atropinized guinea pigs, though sick, recovered from the fatal dose. The colon toxin killed the previously atropinized animals, the atropin apparently not protecting. With frogs both toxins made the frogs sick but the peptone toxin did not kill. Thirty milligrams of colon toxin killed, but previously atropinized frogs survived this amount, though 60 mg. killed in spite of atropin. Apparently atropin possesses in some degree a protective action against the protein poisons but not to the extent to which it antagonizes neurin. Parenthetically it may be of interest to state that if guinea pigs receiving fatal doses of poison are immediately and completely etherized and kept under the ether for some 15 minutes, they show no sign of toxin poisoning.

Cleavage for Monoamino Acids. On the whole everything has seemed to point to protein as the nature of the poisonous group itself though undoubtedly protein of much simpler structure than that of the unbroken molecule. Accordingly attention was turned to an investigation and the isolation of certain protein decomposition products by acid hydrolysis, namely, the

monoamino acids. The poisons selected for this work were those from tuberculosis, typhoid and colon germ substances and for comparison that from egg albumen. For the tuberculosis, the poison from 900 grams germ substance was used—296 grams; for the typhoid 100 grams of the poison; for the colon that from 300 grams of germ substance estimated as 61.5 grams; while for the albumen that from 200 grams of the protein was employed yielding, it was estimated, 93 grams toxin. These poisons were hydrolyzed by boiling under a reflux condenser for 14 hours with concentrated hydrochloric acid (sp. gr. 1.19). The nitrogen of each extract was determined by the Kjeldahl method using an aliquot part of each as a sample and giving the following results:

TABLE 15.

Nitrogen of Acid Extract of Poisons.

Source of Poison.	Per Cent N.
Tuberculosis	9.895
Typhoid	10.38
Colon	10.185
Egg Albumen	11.477

From this point Fischer's (41) ester method for obtaining the individual monoamino acids was carried out. This method is so well known that it is not necessary to outline it here other than to say that after the amino acids have been produced by cleavage of the protein with concentrated acid, the hydrochlorid of their ethyl esters is formed and later the free esters are separated by distillation in the highest possible vacuum. These are then saponified and the amino acids crystallized and purified. The efficiency of this method depending in large measure upon the vacuum secured, the yields here presented for cleavage of the poisons might have been materially increased with a better vacuum, the highest one possible with the apparatus at hand varying from 20 to 30 mm. The following table shows the results of the distillations of the free esters, both the bath and vapor temperatures being given, the amount of the distillates and the yield of crude crystals after saponification.

TABLE 16.

Distillation of the Esters of the Monoamino Acids from Protein Poisons.

Tuberculosis Poison.

FRACTION.	TEMPERATURE OIL BATH.	TEMPERATURE VAPOR.	AMOUNT OF DISTILLATE.	WEIGHT OF CRUDE CRYSTALS.
	degrees	*degrees*	*c.c.*	*gms.*
1	40–60	20–40	5	2
2	60–80	40–60	5	2
3	80–100	60–80	25	16
4	100–130	80–100	25	16
5	130–160		25	7

No distillate passed over between 20° and 95° inside temperature, or between 110° and 138°.

Typhoid Poison.

1	25–60	10–20	12	0.3158
2	60–80	20	8	1.2558
3	80–110	20	2	
4	110–130	95–108	7	6
5	130–145	108–110	4	
6	145–200	138–185	4	

The yield from the colon poison was exceedingly small due to the fact that at one stage of the process part of the solution was lost.

Colon Poison.

FRACTION	TEMPERATURE OF BATH	TEMPERATURE OF VAPOR	AMOUNT OF DISTILLATE	WEIGHT OF CRUDE CRYSTALS
	degrees	*degrees*	*cc.*	*gms.*
1	40–60	28–41	1.5	0.07
2	60–80	41–56	1.0	0.06
3	80–104	56–84	4.0	2.00
4	104–120	84–88	2.0	0.63
5	120–160	88–139	5.5	

Albumen Poison.

FRACTION	TEMPERATURE OF BATH	AMOUNT OF DISTILLATE	WEIGHT OF CRUDE CRYSTALS
	degrees	*cc.*	*gms.*
1	40 – 60	5	0.2638
2	60 – 80	3	0.3338
3	80–100	8	4.000
4	100–130	10	6.000
5	130–160	7	7.000

After repeated recrystallizations these crude products were obtained in a state of chemical purity. From the tuberculosis poison, fractions 1 and 2 yielded needle shaped crystals, soluble in water and alcohol, sweet to the taste and containing 15.773 per cent of nitrogen, the average of four determinations by the Kjeldahl method. Alanin, $C_3H_7NO_2$, has all these properties and contains 15.73 per cent of nitrogen, thus identifying the crystals as alanin. Fischer, Fränkel and others do not give the melting point for *d*-alanin saying that it is not sharp due to the presence of a mixture of the optically active and the racemic forms. The melting point of the crystals from the tubercle poison varied from 268° to 280° corrected, showing no constant temperature. In fractions 3 and 4, the crystals were beautiful, shiny, satiny plates, sweet to the taste, soluble in water, but almost insoluble in alcohol. These sublimed readily, melted with decomposition, and contained 11.976 per cent of nitrogen (average of eight determinations). These properties and the nitrogen correspond with valin, α-aminoisovalerianic acid, $C_5H_{11}NO_2$ which contains 11.965 per cent nitrogen. Fränkel gives the melting point of valin as 298° corrected; when heated in a closed tube, decomposition taking place at the same time. The valin from the tubercle poison melted as high as 296.28° corrected, but after continued recrystallization the melting point dropped as low as 285° and was never reliable. Whether this was due to a partial racemization on repeated heating is not known. Heated in a closed tube the melting point of the final product was 275.8°–278.2°. As is well known valin closely resembles leucin in its properties so that it is very difficult to demonstrate the existence of one in the presence of the other. On p. 542, by another method the

presence of leucin in the poisons has been shown but by the Fischer method of ester distillation valin seems to be the one obtained. The presence of leucin was further demonstrated by the fact that from the final residue left after the esters had been distilled crystals of its decomposition product, leucinimid were obtained. This crystallized from dilute alcohol in the form of needles and melted at 295.4°. Cohn gives the melting point of leucinimid as 295°, Fränkel as 262°. From fraction 5 of the tubercle poison a qualitative test only was obtained for phenylalanin, the quantity obtained being too small for complete purification. After evaporation of the ethereal solution of the thick oily ester, according to the method, the ester is saponified by twice evaporating with hydrochloric acid. It is then evaporated with ammonia, dissolved in a small amount of water and poured into a large volume of absolute alcohol, which precipitates the phenylalanin. From this precipitate the qualitative test was obtained, according to Fränkel (42), by dissolving in dilute sulphuric acid and adding an excess of potassium dichromate, producing the characteristic odor of phenylacetaldehyde and showing thus the presence of phenylalanin. From fraction 5, after removal of the phenylalanin, upon saponification with barium hydrate, there were obtained after the barium had been removed, rhombic, hemihedral crystals which had a distinctly sour taste. These, after purification, showed 9.54 per cent of nitrogen, the average of two Kjeldahl determinations, identifying them as glutamic acid, $C_5H_9NO_4$, which has 9.52 per cent of nitrogen. Fränkel gives the melting point of glutamic acid as 202°–202.5° or quickly heated 213°, with decomposition. The product above obtained melted in an open tube 242°–245°, in a closed tube 236°–238°.

From fraction 1 of the typhoid poison was obtained alanin with characteristic properties as described above. These crystals showed 15.633 per cent of nitrogen and melted 267°–271° Fractions 2, 3, 4 and 5 contained only valin, which showed 11.932 per cent of nitrogen, the average of four determinations. This melted 287°–290.6° in a open tube, 278°–280° in a closed one. From fraction 6, the qualitative test for phenylalanin was obtained as from the tubercle poison.

Owing to the small yield of esters and crystals, fractions 1 and

2 from the colon poison could only be determined qualitatively. Both fractions however showed needle shaped crystals and a sweet taste which in conjunction with the temperature at which their esters distilled indicated alanin. Fractions 3 and 4 gave characteristic valin crystals containing 11.942 per cent of nitrogen and melting at 283.4°–285° in an open tube or 274°–277° in a closed tube. Phenylalanin was obtained qualitatively from this as from the two preceding poisons. After its extraction from fraction 5, and after saponification with barium hydrate and its removal, crystals in the form of rhombic plates and prisms, insoluble in alcohol, were obtained. These correspond with those of aspartic acid, and as the quantity was not sufficient for purification by recrystallization, the copper salt was formed with copper acetate. This was obtained in the form of needles, very difficultly soluble in cold water, difficultly in hot, which again correspond with the properties of aspartic acid.

When the crystals from the fractions obtained from the albumen poison, were examined the result was not different. Fractions 1 and 2 produced characteristic alanin crystals with 11.75 per cent of nitrogen, the average of four determinations. The melting point was 277°–279.6°. Valin, with form and properties as already given was obtained from both fractions 3 and 4, containing 11.935 per cent of nitrogen, and showing a melting point of 282°–286.4° in an open tube, and of 279°–283° in a closed tube. Likewise from fraction 5, the heavy oil of phenylalanin ethyl ester was obtained and from this as in the other cases the qualitative test for phenylalanin by the production of phenylacetaldehyde. The remaining portion of fraction 5 yielded the same rhombic plates and prisms as described under the colon fractions and which are like those of aspartic acid properly obtained at this point if present. The copper salt was again formed, the same needles, very difficultly soluble in cold water, difficultly in hot, being obtained. The amount of crystals was too small for further identification.

From this it will be seen that monoamino acids are obtained from the protein poisons after hydrolysis with strong acid. It is not claimed that these are the only monoamino acids present or that all of these have been sufficiently identified, but in consideration of the fact that those disscused were found in the

proper fraction according to Fischer's separation and according to the boiling points of their esters, that the crystalline form and qualitative properties corresponded, and that, when it could be determined, the percentage of nitrogen was close to the theoretical it seems fair to conclude that the following tabulation is not far from correct.

TABLE 17.

Monoamino Acids of the Protein Poisons.

TUBERCULOSIS POISON.	TYPHOID POISON.	COLON POISON.	ALBUMEN POISON.
Alanin	Alanin	Alanin	Alanin
Valin	Valin	Valin	Valin
Phenylalanin	Phenylalanin	Phenylalanin	Phenylalanin
Glutamic Acid		Aspartic acid	Aspartic acid
Leucinimid			

This is sufficient to establish the point for the proof of which the method was employed, that is the protein nature of the poisonous group of the protein molecule. Attention is called also to the comparative simplicity of the group and to the great similarity of acids obtained from the different poisons. This accords well with the great similarity and non-specificity of their physiological action.

It is interesting that the final residue left after distillation of the esters gives still a very intense Millon reaction, which cannot be ascribed to the presence of tyrosin.

To recapitulate briefly, the bacterial cellular substances obtained in large quantity freed from non-poisonous alcohol-ether extractives, are of complex but definite protein composition and are highly poisonous whether derived from pathogenic or non-pathogenic bacteria. They respond to all the protein color reactions. Physical solvents scarcely affect them but they are partially digested by pepsin and trypsin with a lessening of their poisonous properties. Both dilute and strong mineral acids effect definite cleavages, which show the chemical nature of the whole protein, but do not separate the poisonous group in a free form. This is accomplished by hydrolysis with

dilute alkali, best in alcoholic solution, which leaves the non-poisonous portion undissolved. The process is accompanied by the production and loss of ammonia. The non-poisonous portion of the cellular proteins shows most of the protein color reactions, contains all the carbohydrate of the unsplit molecule, most of the phosphorus and is the specific part of the cell, the part that immunizes and sensitizes. The poisonous portion, freely soluble in absolute alcohol, shows all the protein color reactions except the carbodydrate test. It is highly poisonous, killing in less than an hour, but has no specificity in its action, the symptoms being identical whatever the protein from which the poison is taken. It has not been possible to obtain any poisonous base or to isolate the toxin as a chemical unity, but from the monoamino acids obtained after its hydrolysis with concentrated acid it is concluded that the poison itself is protein in nature, though simpler in structure than the complex proteins of the bacterial cells themselves.

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